THE EFFECTS OF SOME ENZYMES ON INTERCELLULAR ADHESION

by

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SUMMARY

The formation of adhesions by cells of a permanent line derived from baby hamster kidney tissue (BHK/clone 13) has been investigated by aggregating the cells in suspension in a medium composed of glucose and salts. The initial single cel suspensions were produced by the use of trypsin to disperse cells grown in monolayer culture.

The kinetics of aggregation was found to fit a rate equation in which the total particle concentration decayed exponentially from its initial value to a stable final value. This limiting final population contained both single cells an clusters of various sizes. Aggregation was followed by using the Coulter counter to measure the particle concentration dur the process. Best fit curves and statistical information wer obtained from the data using a KDF-9 computer. The aggregati of any cell suspension could be described by two parameters, rate constant and final extent of aggregation.

The effect of trypsin on adhesion was examined by treating cell cultures, and fresh suspensions with different concentrations of the enzyme before aggregation. In additio trypsin was applied to pre-formed cell clusters. C-13 aggregation was progressively reduced by the application of increasing amounts of trypsin. Trypsin and pronase were found/

found to be similarly effective in dispersing C-13 clusters but collagenase, phospholipase C and EDTA (at pH 7.2) had no detectable effect on C-13 aggregation. It is concluded that protein or glycoprotein elements of the cell surface are important for adhesion in this system.

The effect of the removal of cell surface neuraminic acid was studied either by 1) adding neuraminidase to suspensions of pre-formed aggregates or fresh cell suspensions as they began aggregating; or 2) by pre-treating cell cultures before aggregation. The activity of the neuraminidase was tested by a fluorimetric assay in which the neuraminic acid released from the cells by the enzyme was treated with neuraminic acid aldolase, to yield pyruvate. The pyruvate formed was measured by using it to oxidize NADH in the presence of lactate dehydrogenase.

The results showed that neuraminidase treatment increased aggregation by as much as 50% above the controls. This effect may be understood in terms of a decrease in the cell surface net negative charge or by a requirement for the newly exposed terminal sugar groups (formerly bound to neuraminate) in cell adhesion.

It was found that complex media (e.g. Eagles medium) also caused an increase in the rate and extent of cell aggregation. The kinetics of the effect was different from that /

that mentioned above. The components of Eagles medium were tested for their activity and it was found that only the amin acid L-glutamine caused aggregation. The effect was inhibited by NaF and by the specific analogue of L-glutamine, azaserine. L-glutamine is thought to function by supplying amino groups to cell surface amino sugars which may be directly required for intercellular adhesion.

The results are discussed in terms of the known molecular composition, structure and metabolism of the cell surface.

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CONTENTS

EXPERIMENTAL

I. GENERAL INTRODUCTION

Throughout the fertilization, development and adult life of animals, the surface of each cell is in a position of paramount importance to mediate or affect vital cellular interactio and behaviour. Studies of cell behaviour *in vitro* have led to the description of a number of phenomena, each thought to be relevant to processes important in morphogenesis. These phenomena include the sorting out of cell types (Steinberg 1958 the formation of low resistance junctions between cells (Furshpan and Potter 1968), Topoinhibition (Dulbecco 1970), fibroblastic locomotion (Abercrombie 1966) contact guidance (P.Weiss 1941)and contact inhibition of locomotion (Abercrombie and Heaysman 1954). I have mentioned these particular phenomen since it has been constantly suggested in the literature that none of them can be expressed unless cells develop contact, probably adhesive contact,(Abercrombie 1966). Therefore a thorough understanding of these phenomena and of morphogenesis must include understanding of intercellular adhesive behaviour.

The composition and structure of the cell surface are thought to be of primary importance in regard to intercellular adhesive behaviour as well as to the phenomena listed above (Kalchar 1965, Curtis 1967, Wallach 1968). It is the purpose of this study to examine the part played in an adhesive interactio of a particular cell type by some of the molecular components of /

of the cell surface, using specific enzymes to modify the surface.

REVIEW

II. The Nature of the Surface of Animal Cells.

A. Composition of the plasma membrane

In order to form a more specific conception of the cell surface, I propose to review the nature of the plasma membrane (PM). It is possible that the surfaces of certain animal cells may be composed of a coat (much like that of the egg) which is distinct from the PM in molecular composition and structure. More typically perhaps the properties of the cell surface may be those of the outer face of the PM. The distinction between these two possibilities may often be largely a matter of choice of operational definition of the plasma membrane.

Most workers have used myelin or erythrocyte material which have yielded several principles probably of general applicability to all animal plasma membranes. PM from less specialized tissue cells and cultured cells, although very fragile, has been studied only since the advent of improved isolation techniques which stabilize the membrane (Warren et al 1966, Stoeckeneus and Engelman 1969). The purity of almost all PM preparations remains an open question. Components of the medium or of the cytoplasm may absorb onto the PM, or conversely the membrane may lose some of its elements through the procedure of PM isolation (see Stoekineus and Engelman, 1969, for a review).

1) PM lipid composition/

1) PM lipid composition

Since the work of Danielli and Davson (1934-35), other workers have confirmed the major structural and functional roles of lipid in PM models (Wallach and Zahler 1966, Lenard and Singer 1968, Glaser et al 1970). In addition many, but not all, of the non-specific permeability and electrical properties of natural plasma membranes are qualitatively similar to those of synthetic lipid bilayers (see Finean 1966, Tien and Dana 1968). In animal cells, only a small fraction of the total cellular lipid is contained in the PM but this makes up as much as a third of the total PM weight (Benedette and Emmelot 1968, Rouser et al, 1968, Weinstein et al 1969).

Four lipid classes are almost always present in the PM of animal cells: sterol, phospholipid, sphingolipid and glycolipid. The sterol is probably always cholesterol (Fleischer and Rouser 1965, Ashworth and Green 1968). In preparations of PM studied so far, phospholipid has accounted for about 80% of the total lipid with lecithins the major component (see Weinstein et al, 1969). Phosphatidyl-serine, -ethanolamine and inositol along with sphingomyelin, lysolecithin, phosphatidic acid and triglyceride have also been measured as normal PM components (Rouser et al 1968, Weinstein et al 1969). It is possible that at least the last named lipid may have originated from the cytoplasm during membrane isolation. Klenk and Choppin (1969) have characterized in detail the PM and whole

cell/

cell lipids of a BHK-21/C13 variant. Lecithin was the major phospholipid and the lipid composition was generally similar to that of erythrocytes (de Gier and van Deenen 1961, Benedetti and Emmelot 1968, Weinstein et al 1969), liver and L cells.

The relative proportions of the lipids in any one cell type seems fixed. Plageman (1968) found that the density of hepatoma PM remained unchanged after growing the cells in extremes of choline concentration. However, for at least some fatty acid chains this is not the case. The fatty acids incorporated into the PM depend to some degree on diet since some cells cannot synthesize all the varieties they require. If a particular fatty acid is missing another may be substituted in its place in the lipid (Chapman and Wallach 1968, Klenk and Choppin 1969).

A number of workers have shown that the lipid pattern of the PM differs from that of the internal membranes (see Rouser et al 1968). This is also true of BHK cells (Klenk and Choppin 1969). Glycolipids usually exist as either cerebroside and sulfatide or as ceramide polyhexosides and gangliosides in any one cell but the total amount is only about 1% of the total PM lipid (Weinstein et al 1969). Rouser et al (1968) have pointed out that virtually all cellular glycolipid is located in the PM. They have also summarized the lipid analyses of many cell types and find that, unlike the /

the other lipid classes, glycolipid composition differs between species and between cell type. Glycolipids are discussed further in a later section with emphasis on their sugar patterns

2) PM RNA

Carbohydrate amounts to about 3% of the weight of the PM of many cell types (Weinstein et al, 1969, Benedetti and Emmelot 1968). Most, if not all, is bound as glycolipid or glycoprotein. However, Warren and Glick (1968) believe that L cells may contain RNA associated with the PM in functional ribosomes (see following). The ribosomes were attached to the inner side of the PM. They could be isolated with the PM using several different isolation techniques. Weiss and Mayhew (1967) suggested on the basis of electrophoretic studies that some cells have cell surface RNA. The authors have not considered the possibility that the RNA may originate from the cytoplasm of lyzed cells.

3) PM protein composition

There are two difficulties in interpreting protein analysis of PM preparations. Firstly, protein associated with isolated PM may not necessarily have originated from the PM. Warren and Glick (1968) find that the PM of L cells contains only 3 - 5% of the total cell protein so that contamination is an important possibility. Secondly, protein may be associated with subsidiary structures such as ribosomes. desmosomal/

desmosomal and actin-like filaments attached to plasma membranes (Glick and Warren 1969, Giacomelli et al 1970). Some glycoproteins have been identified as PM components chiefly because they are enzymically accessible at the cell surface. Similar glycoproteins have been isolated from intact platelets (megakaryocyte fragments) and erythrocytes (Pepper and Jamieson 1968). An N-acetyl-neuraminic acid (NANA) containing glycoprotein from the surface of Novikoff ascites cells was isolated and analysed by Walborg et al (1969). Humphreys (1970) has also isolated and examined the ultracentrifugation properties of a glycoprotein from some sponges. He believes the molecule may coat the cell surface. Glycoproteins of the cell surface are reviewed in more detail later.

Numerous enzymes have also been reported as components of the PM (see Benedetti and Emmelot 1968, for a review). Gahmberg and Simons (1970) have analysed some of the enzymes in the PM of BHK cells including Na-K-ATPase, 5'-nucleotidase, acid phosphatase, β -glucuronidase and NADH-diaphorase. Two reports of nucleotide-sugar transferases located at the cell surface may have important implications (see later). Evidence has been found for a specific UDP-galactosyl transferase at the surface of chick embryonic neural retina cells (Roseman 1970 and a collagen glucosyl transferase in PM preparations from HeLa cells (Bossman and Eyler 1968).

Glick/

Glick and Warren (1969) have found that isolated PM of L cells is capable of synthesizing and incorporating distinctive protein. The amount of protein synthesized by the PM was 10-fold greater than that produced by other microsomal fractions. Most importantly, electrophoresis on acrylamide gels showed that the protein products of the PM were quite different from those of any of the other membrane fractions. Using electron microscopy, the authors located ribosomes firmly attached to a "fuzz" on the cytoplasmic face of the isolated or intact PM. This material was found regardless of which of several PM isolation techniques was employed. The results led the authors to suggest that such an arrangement of metabolic machinery might be sensitive to cellular contact interactions.

B . Plasma Membrane Metabolism

The PM can no longer be regarded as a chemically stable entity. Collectively, the metabolic studies of Warren and Glick (1968) and Bossman and Winston (1970) suggest that the components of the PM of non-growing cells are turning over while during growth there is a net incorporation of new membrane into the PM with some slight degree of turnover. By following the fate of isotopically labelled protein, lipid and sugar elements in the PM, Warren and Glick (1968) and Warren (1969) concluded that the rate of incorporation of new PM was similar in/

in growing and non-growing cells, but that in the latter there was an equivalent rate of PM degradation. The authors also found that there was no differential turnover of the protein, lipid and sugar of the PM.

In "chase" experiments, similar to those of Warren and Glick (1968), Pasternak and Bergeron (1970) found evidence which conflicts with the conclusions of Warren (1969) and Bossman and Winston (1970). Using neoplastic mast cells, Pasternak and Bergeron (1970) followed the fate of isotopically labelled choline, inositol, glycerol, acyl chains, valine and thymidine. They interpreted their results to mean that most of the lipid turns over at the same rate whether the cells are growing or not. A minor lipid fraction was stable. All cellular fractions displayed the same rate of turnover. Most importantly they found that phosphoryl choline was more unstable than was sphingomyelin and that the unstable lipids turned over at a much greater rate than did either protein or DNA.

Bergeron et al (1970) suggested that lipid (choline) was incorporated into the PM during the "S" growth phase. Warren (1969) found that both protein and sugar also were incorporated during the "S" phase. However, Bossman and Winston (1970) claimed that lipid and glycolipid were incorporated during the "G_2" and "M" phases. If the rates of

PM/

PM incorporation and degradation during turnover are equivalent (Warren and Glick 1968) the proposal of constant turnover is not consistent with that of periodic increases in incorporation.

Pasternak and Bergeron (1970) find that 90% of the radioactivity released during turnover from labelled cells is water soluble and they suggest that a phospholipase is responsible for the selective turnover of the membrane phosphoryl choline. However, there is no evidence for this mechanism and other possibilities have been suggested (see following).

The authors have rejected the possibility that the constant turnover is characteristic of neoplastic cells since Pasternak and Friedrichs (1970) found similar turnover in rat tissues.

Kraemer (1967) has measured the metabolic regeneration of NANA on the surface of neuraminidase (NANase) treated cells from several sources. A small fraction of NANA resisted enzymic removal (see later). The total amount which is removed is regenerated over a period of 12 to 16 hours (approximately one cell cycle). Both the rate and extent of replacement were found to be independent of the manner in which the cells were grown, i.e. on glass or in suspension (Kraemer 1966). The total amount of cell surface NANA differed between species but was proportional/

proportional to the estimated surface area of the cells. This ratio remained a constant value during all phases of the cell cycle. Marcus and Hirsh (1963) found similar regeneration kinetics for NANA on HeLa cells after the use of NANase. Unlike Kraemer (1966) the authors found that actinomycin D had no effect on NANA regeneration.

On the other hand, Shen and Ginsberg (1968) found that monolayer grown cells had a higher amount of heteroglycan than did suspension grown cells. Hakamori (1970) has suggested that this difference is due to the higher degree of intercellular contact in monolayer grown cells. Using non-malignant BHK-21/C-13 cells and a diploid human fibroblast (8166), Hakamori (1970) found that the levels of some of the glycolipids (including galactosylgalactosylglucosyl -, N-acetyl-neuraminylgalactosylglucosyl -, and (N-acetyl-neuraminyl)$_2$ galactosylglucosyl -ceramide) were increased as the monolayer culture grew denser and intercellular contact increased. Meezan et al (1969) has found a similar effect of density or contact on the sugar pattern of mouse cells in culture.

The Golgi region is thought to be the site of addition of carbohydrate residues onto newly synthesized PM before it is inserted into the cell surface (Peterson and Leblond 1964, Hicks 1966, Peterson and Rubin 1969).

The /

The PM may be incorporated at particular sites on the cell surface. Using haemagglutination techniques, Marcus and Hirsch (1963) and Marcus (1965) described the appearance of NANA containing, viral receptor sites on viral antigens into the surface of normal or infected HeLa cells. Incorporation begins at one or both poles of the cell and increases in area until, after one growth cycle, the entire cell surface is covered. Abercrombie et al (1970) have suggested that new fibroblast cell surface erupts as lamellipodia (formerly called ruffled membrane) at the leading edge of the cell.

Lipid, protein and sugar macromolecules alone, or perhaps as whole pieces of PM, are believed to be shed into the medium and interfaces surrounding cells in culture (Kraemer 1966, 1967; Bossman and Winston 1970; Warren 1969). Peterson and Rubin (1969) found that chick embryonic fibroblasts released phospholipid or lipoprotein into the medium as a function of the serum concentration. They believe that these macromolecules originate from live cells and are likely to establish an exchange equilibrium between cell and medium or intercellularly. Kite and Merchant (1961) found that L cells, previously grown in media containing horse serum, were capable of binding significant amounts of anti-horse globulin to/

to their surface even after washing. Other workers have found that several cell types absorb antigens from the medium onto their surface (Hamberger et al 1963, Kodani 1962).

Warren (1969) has suggested that the origin of shed material may be as pieces of PM sloughed off cells. In neoplastic cells this phenomenon might be exaggerated and arise from an error in the degradative process of PM turnover leading to an accumulation of old PM. By masking the cell surface, Warren thinks old PM might affect the histology and especially the contact behaviour of malignant cells (see section on Cell Coats below). However, the possibility that all of these materials originate from lysed cells has not been eliminated.

C. PM and Cell Surface Structure

Recent information makes it necessary to revise the model of the PM as a lipid bilayer coated with protein which was first suggested by Danielli and Davson (1934-35). Therefore in this section I intend to review some current models of the PM structure, especially as they affect concepts of the cell surface. Some authors have contended that the cell surface consists of an essentially mucopolysaccharide coat and this idea is also discussed.

Studies/

Studies of the physical properties of plasma membranes have confirmed the existence of the lipid bilayer but suggest new ideas about the structure of PM protein and suger. It also seems theoretically possible that the PM may undergo changes from one configuration to another and these may be of importance in contact interactions.

Much of value in interpreting the structure of the PM has been obtained by studies of synthetic membrane model systems composed of lipid or protein and lipid. The sensitivity of the PM to chemical or physical manipulation has limited the usefulness of many techniques of study. In particular, the dehydration of PM material in electron microscopy may lead to permanent alteration of PM structure as may normal fixation and staining procedures.

1) PM structure: lipid models

Using mixtures of lipid and protein, Lucy and Glauert (1964) concluded that lipid in water suspensions adopted either one of two basic configurations primarily as a function of the water concentration. Where water was limiting micelles were formed with the charged phospholipid heads grouped about drops of water. With an excess of water, bilayer structures were formed; that is, two monolayers of lipid sandwiched with their hydrocarbon chains together and their heads in the medium./

medium. The authors concluded that biological membranes ought to be freely capable of adopting either configuration unless inhibited by other membrane components. The configurations of maximum thermodynamic stability of lipids in vitro are thought to be models for their configuration(s) in the PM (Lucy 1968).

Electron microscopy of freeze-cleaved preparations of several types of cells and membranes show that the membranes split along which is best interpreted as the interior hydrocarbon region of the lipid bilayer (see Chapman and Wallach 1968). In addition, many of the electrical properties of synthetic membrane bilayers resemble those of natural PM (Chapman and Wallach 1968, Finean 1966).

It would then seem clearly inevitable to propose that the lipid containing envelope of the cell exists as a lipid bilayer in accordance with Danielli and Davsons original model. In many ways, it is yet the most acceptable of the models proposed for the PM (Stoeckeneus and Engelman 1969).

It is possible that while some or most of the PM exists as a bilayer, the rest is structured as micelles or macromolecular subunits of lipid and protein. Electron microscopy of negatively stained preparations of PM by Benedetti and Emmelot (1968) and lanthanum staining PM sections by Revel and/

end Karnovsky (1967) show geometric arrangements over limited areas of the PM, thought to be part of tight junctions, (see later).

The most cogent evidence for the existence of the bilayer in plasma membranes comes from freeze-cleave investigations. Branton (1966) has suggested that this technique splits the cell membrane along the plane of the hydrophobic fatty acid interior where Van der Waals forces are likely to be weak. Deamer et al (1970) carried out studies of freeze-cleaving of lipids. Their results indicated that the bilayer structure of a lipid at high temperature was preserved upon freezing as were the hexagonal (micelles) structures. This was found using oleic acid. Slow freezing of this lipid resulted in crystalline plane formation.

Egg lecithin, alone or mixed with various concentrations of cholesterol and water, fractured along the non-polar region. The degree of acyl saturation, hydration or cholesterol concentration had no effect on fracturing.

2) Structural changes

Reversible transitions of PM between micelle and bilayer forms may be possible if its lipid-protein structure is sensitive to changes in the ambient temperature, ion concentration or electric field. Such a structural alteration may act as a functional response of the cell to a new situation and/

and any specific concept of cell surface structure must take this possibility of transition into account (Gingell 1967). Theoretical conditions capable of initiating phase changes have been listed by Gingell (1967), Changeux et al (1967), Changeux and Podleski (1968) and Hill (1967). These conditions include ionic strength, molecular adsorption and the proximity of charged molecules and surfaces (e.g. plasma membranes) (Gingell 1967).

Kavanau (1965) has proposed that the PM may be capable of exhibiting several configurations and he considers that calcium ion-carboxyl group interactions may play a leading part in controlling conformational stability. There is as yet little or no direct evidence for this hypothesis. However, Loewenstein (1968) suggests that intercellular electrical contact through low resistance junctions involves alteration of PM permeability coincident with loss of calcium from the membrane. Benedetti and Emmelot (1968) believe the tensile strength of the PM to be a function of bound calcium.

Shah and Schulman (1967) have related the membrane's lipid composition to its capacity for ionic interaction. Calcium ions are bound by the phosphates of adjacent phospholipids in/

in lipid monolayers and by doing so increase the electric surface potential of the monolayer. Zull and Hopfinger (1969) examined the effects of lipid composition on the calcium ion binding capacity of lipid monolayers. Their results led them to suggest that the configurational differences between the head groups of sphingomyelin and lecithin are the cause of significant differences in their ion binding values. Also, ethanolamine monolayers seem capable of stronger interactions with anions compared to monolayers of choline containing lipids. The authors believe these findings may have some bearing on cellular contact interactions.

Gottshalk (1960) has suggested that glycoproteins carrying many acid groups on their surface (e.g. NANA) owe their conformational rigidity to repulsion of the charged groups by one another. Therefore, PM - ion interactions might function similarly.

3) Liquid-crystalline state

The plasma membranes of Mycoplasma sps., which lack cholesterol, undergo phase changes of the acyl chains as a function of temperature as detected by calorimetry (Steim et al, 1969, Reinert and Steim 1970). Cholesterol has been suggested to modulate the fluidity of the lipid hydrocarbon chains effectively producing an intermediate fluid state at biological/

biological temperatures (Chapman and Wallach 1968). Using stereomodel projections, Vandenheuvel (1963) found close fit between models of cholesterol and myelin sheath lipids. There is a positive correlation between phospholipid and cholesterol ratios in most PM (Weinstein et al 1969). Finally, using circular dichroism and proton magnetic resonance techniques, Glaser et al (1970) found that erythrocyte PM, which usually has a large amount of cholesterol, showed little temperature sensitive acyl chain mobility.relative to that of Mycoplasma PM. Finean (1953) suggested a high cholesterol-phospholipid ratio might promote order and rigidity in membranes. Klenk and Choppin (1969) support this view and find that the PM of one cell type derived from monkey kidneys, has a ratio of 0.81 and is more resistant to stress than is that of the BHK-21/C13 cell which has a ratio of 0.68. Nevertheless, nuclear magnetic resonance studies by Chapman et al (1967) indicate that PM lipids from erythrocytes, suspended in water, exist with considerable acyl chain mobility similar to that of the same lipids in chloroform. This is consistent with a bilayer configuration of the lipid as was the authors' finding that the hydrophilic choline and sugar groups were also mobile.

4) PM/

4) PM Lipid-protein structure

The Danielli-Davson model of the membrane proposed that lipid-protein relationships occurred through extensive electrostatic interaction of the charged phospholipid heads with all of the protein adsorbed at the interface of the lipid bilayer. The familiar triple-layer appearance of membranes in section by electron microscopy has been used to support this model (Stoekineus and Engelman 1969).

Recent physical studies of plasma membranes using circular dichroism, optical rotary dispersion and proton magnetic resonance techniques have been applied to the construction of PM models more consistent with the conclusion that the majority of PM protein is interacting hydrophobically with the lipid. Wallach and Zahler (1966), Lenard and Singer (1966, 1968a) and Glaser et al (1970) have independently suggested that the polar groups of lipid and protein in the PM are located in contact with the external medium and that much helical protein exists stabilized in the hydrophobic interior of the membrane. Wallach and Zahler (1966) further suggested that some aqueous channels lined with polar groups may form across the PM. Glaser et al (1970) believe a pattern of protein-lipid mosaic may exist. However, the spectra of PM material by both circular dichroism and optical rotary dispersion/

persion are anomalous in the sense that the spectra are unlike what is expected of normal protein configurations (Chapman and Wallach 1968). Using calorimetric methods, Steim et al (1969), Reinert and Steim (1970) and Tourtellotte et al (1970) found that at least 70% of the lipid of Mycoplasma sps. PM was capable of participating in a thermal phase change (whether in vivo, as isolated PM or if reconstituted from a solvent extract of PM). Reinert and Steim (1970) suggested that the lipid fraction which remained stable was in association with the membrane protein.

Electron microscopy of plasma membranes in section has usually shown a trilayer structure with two, electron dense outer layers of 20-30 Å thickness and an inner layer of low electron density of 30-40 Å (Benedetti and Emmelot 1968). The structure is revealed especially when $KMnO_4$ or OsO_4 is used in fixation and staining. The trilayer pattern has been taken as evidence of the Danielli model of a symmetrical membrane bilayer (see Stoekineus and Engelman 1969). Robertson (1966) suggested that the dense lines represented the polar regions of lipid and protein, and the low density line that of the hydrocarbons. However - Korn (1966, 1967) found that OsO_4 reacted with the hydrocarbon region of the lipid and concluded that little information about lipid configuration could be forthcoming from/

from EM staining with OsO_4. Lucy and Glauert (1964) observed that OsO_4 induced a change in lipid structure in lipid micelles. Lenard and Singer (1968b) found that commonly used EM fixatives, especially $KMmO_4$, caused changes in PM protein configuration. Lesseps (1967) treated lanthanum and $KMmO_4$ stained chick embryonic cells with phospholipase C. Only this enzyme was effective in removing the lanthanum staining layer on the cell surface. Other enzymes including proteases were not effective. Interestingly, the normal trilayer appearance of the PM was revealed after phospholipase C treatment which may indicate that the $KMmO_4$ staining was other than at the phosphoryl choline moiety.

There seems little evidence for cell surface protein based on EM staining evidence (Korn 1967) or immunological evidence (Kite and Merchant 1961). However, enzymic techniques have given a reasonable demonstration that protein exists at the cell surface and this point will be discussed in the following section.

D. The Nature of the Cell Surface

1) The concept of the cell surface

A useful conceptual distinction between the PM as a whole, and the cell surface has been put forward by Curtis (1967). i) The/

The PM, as a trilayer structure, may be composed of an inner region of low dielectric constant and high electrical resistance. ii) This region is enclosed by a region of high dielectric constant and low resistance. In an aqueous environment, interactions with (hydrophilic) molecules or cell surfaces, would probably be limited to occurring with components of the outer, hydrophilic region, the impermeable interface offered by the hydrophobic inner region (i) would tend to seriously inhibit interactions from extending deeper, beyond this surface. Effectively, the properties of the outer region would also be the properties of the cell surface. It is the concern of this section to discuss the element of Curtis' definition in molecular terms.

Several points of uncertainty must first be examined. a) Not all molecular groups in the outer region may be easily accessible, since they may be masked or covered by other chemical groups (Inbar and Sachs 1969b). b) In certain circumstances, the inner region may be penetrable, thereby weakening the PM-cell surface distinction (Finean 1966). c) Not all of the elements of the cell surface may be components of the PM. Some may be adsorbed from the medium or acquired in other ways (e.g. cell coat).

A/

A number of techniques have been used to establish criteria for a definition of the cell surface. Histology, with both light and electron microscopy, immunology, enzymology and electrophoresis of intact cells offer an operational approach to the question of the identity and accessibility of cell surface molecules.

2) Staining methods

Histological methods may be useful in structural analysis if the stain is able to react with only part of the PM, (e.g. the cell surface) being limited by the permeability barrier of the membrane; or if it reacts spcifically with one or few PM components. For operational reasons, it may be assumed that the hydrophobic acyl interior of the PM bilayer and the cellular permeability barrier are identical although, from the discussion above, this must not be taken for granted. General or specific precipitation stains used in structural analysis of the PM may lack resolution required to decide unequivocally the location of the stained molecules.

Several EM studies have used colloidal lanthanum stain, for example, that of Lesseps (1967) discussed previously. Overton (1969) also stained chick embryonic cells with lanthanum, treating both intact tissues and aggregates of dissociated tissue. Interestingly, dense ordered and tangled arrays/

arrays of fibrils (35Å center to center) were found at the cell surface. Unfortunately, no clear relationship between the fibrils and the PM was demonstrated.

Revel and Karnovsky (1967) used lanthanum in an EM study of mouse tissues. The stain revealed hexagonal patterns at areas of "tight junction" contact when viewed normal to the plane of the PM. Only an intercellular gap and the outer leaflet of the PM were stained. The hexagons were 70-75Å wide, 50Å deep with 30-35Å thick walls.

Flaxman (1968) found an electron dense layer of a few hundred angstroms thick between the central PM of fibroblasts and their growth substrate. No such layer was found at intercellular contacts.

Colloidal iron hydroxide (CIH) was used to stain intact tissues and isolated PM of rat liver and hepatoma by Benedetti and Emmelot (1967, 1968). CIH, when used at low pH, is thought by the authors to precipitate specifically with exposed NANA groups. However, there is no reason why other carboxyl groups should not also react. High resolution EM of isolated and stained PM demonstrated that the outer PM leaflet (cell-medium interface) was stained at discreet points at intervals of approximately 200Å. Pre-treatment of the PM with NANase abolished/

abolished all such staining (but for infrequent small patches which occurred on opposite inner and outer leaflets). No staining was obtainable on the inner PM leaflet or at any intercellular junction. After the PM was treated with EDTA, the freshly exposed faces of the loosened desmosomes and intermediate junctions could be stained with CIH. "Tight junctions" remained refactory to EDTA treatment and no NANA could be demonstrated at this junction.

In an EM and light microscope study of rat tissue using periodic acid-silver methenamine and colloidal thorium stains for acidic glycoprotein, Rambourg and Leblond (1967) found that the intercellular spaces of the tissues were filled with stain. The authors suggested that the cells were enveloped in a coat of acidic (NANA containing) glycoprotein. No staining was found at "tight junctions", but other junctions were stained, as was the dense central band of the desmosomes which was continuous with the rest of the intercellular staining layer. The results were interpreted by the authors to indicate that a carbohydrate coat exists surrounding the cells. The resolution of their micrographs does not offer any evidence that the staining glycoprotein was in fact part of a cell coat, distinct from the outer surface of the PM.

Defendi/

Defendi and Gasic (1963) carried out a light microscope study of cultured normal hamster embryo cells and polyoma virus transformed cells staining for acid mucopolysaccharides. They concluded that the transformed cells were thickly coated by acid mucopolysaccharide. However, the same criticism can be applied to their interpretation as to that of Rambourg and Leblond (1967). The resolution of the microscopy was insufficient to identify a distinct cell coat.

3) Accessibility methods

The degree of accessibility of a particular PM component (e.g. to an enzyme or immunoglobulin) might indicate whether or not it is located at the cell surface. Certain enzymes, antibodies and lectins are capable of reacting with their specific substrates or antigens on intact cells. The impermeability of the PM should limit these interactions to the outer PM region of the Curtis cell surface definition (see above). However, substantial exposure of a live cell to any of these large molecules could result in their ingestion by pinocytosis or phagocytosis.

It is conceivable that a large protein might penetrate into the PM or cytoplasm by reason of the thermal motion inherent in the liquid structure of the PM (Finean 1953), or by/

by inserting side chains into the hydrocarbon interior of the PM (Dawson 1968). Possibly an enzyme might digest its way into the PM.

a) Enzymic analysis of the cell surface

Often cells or tissues can be treated with active enzymes such as lipases, proteases or glycosidases without impairment of cellular viability. Of special interest are trypsin, neuraminidase, collagenase, pronase and phospholipase C, which are all used in this study. Proteases are routinely used to harvest cells for culture purposes. Trypsin, the most commonly used enzyme for dispersal of cells, cannot easily penetrate the PM (Kraemer 1967a, Onodera and Sheinan 1970, Allen and Snow 1970).

Substantial amounts of glycopeptides, which can be identified as part of the PM by biochemical studies (Warren and Glick 1968, Meezan et al 1969) are released by the cell following attack by proteases of limited specificity, such as trypsin and chymotrypsin. Sialoglycopeptides have been shown to be liberated by trypsin from erythrocytes (Cook et al 1960, Winzler et al 1967), chick embryonic cells (Kemp 1970), Novikoff ascites cells (Walborg et al 1969, BHK-21/C13 cells (Allen and/

and Snow 1970) and from other cell types (Onodera and Sheinin 1970, Kraemer 1966).

Steinberg (1963) has shown that trypsin treatment of cells releases a gel-like DNA-trypsin complex into the medium. The material is produced by the action of trypsin on dead or permeable cells but not live impermeable cells. (Kraemer 1966, Allen and Snow 1970).

Philipson et al (1965) have shown by an elegant technique that subtilisin does not enter tissue culture cells, although it is active in destroying viral receptor sites on the cell surface. The intra-cellular enzyme lactate dehydrogenase is highly susceptible to destruction by subtilisin attack. No decrease in the activity of the intracellular enzyme was detected after subtilisin incubation with intact cells but 50% of the dehydrogenase from broken cells was inactivated by a concentration of subtilisin which was only 4% of that used to treat the cell surface.

Purified collegenase can be added to celluler culture media with no detectable effect on cell growth or viability although intercellular collagen is digested (Elsdale and Foley 1969). Phospholipase C may be incubated with several growing cell types with no apparent effect on cell viability, RNA or protein synthesis (Friedman and Paston 1968). The

enzyme is able to release all, or nearly all, of the phosphoryl-choline from isolated erythrocyte PM without disturbing the structure or causing loss of any of the other PM components (Lenard and Singer 1968a,b). Pronase, although it may contain as many as four enzymes of differing specificities (Trop and Birk 1970), has been used without harm on ganglia (Banks et al, 1970) and erythrocytes (Houba 1967). Takeuchi and Yabuno (1970) have used pronase and trypsin on slime mold cells with no visible effect on cellular viability.

NANase was found to be unable to penetrate HeLa cells. Although the enzyme released NANA from intact cells NANA on the surface of the nucleus was unaffected (Marcus et al 1965). Nordling and Mayhew (1966), using fluorescien iso-thiocyanate labelled NANase, claimed that the enzyme was able to penetrate erythrocytes. However, since the enzyme was incubated with the cells for 30 minutes, the possibility has not been eliminated that the uptake was caused by pinocytosis. Nacetyl- and Nglycol-neuraminic acids are found at the cell surface by treatment of a variety of cells with NANase (Klenk 1958, Kraemer 1966).

It is conceivable that although an enzyme is unable to attack the cytoplasm of acell it may be able to "roam" and gain access/

access to all of the PM. Phospholipase A is active in cleaving fatty acids from lipids in intact cellular plasma membranes (Fischer et al 1967). In a membrane strictly modelled as a bilayer the acyl chains should be inaccessible to hydrophilic substances in the extracellular medium. It can be demonstrated that neither trypsin or NANase is able to gain access to all parts of the PM. Cell surface NANA exists as a terminal group on glycolipids and glycoproteins, however only the NANA of glycoproteins is generally susceptible to attack by NANase (Klenk 1958, Kraemer 1966). The NANA of gangliosides in plasma membranes seem protected in some way from the enzyme. The isolated glycolipids are readily attacked by NANase.

Glycopeptide fragments from trypsinized human erythrocytes yielded one molecular species of molecular weight 31000 containing NANA, Nacetyl-glucosamine, Nacetyl-galactosamine, fucose and hexose (Winzler et al 1967). Other cells treated with trypsin also released a limited number of glycopeptide species. Onodera and Sheinin (1970) found only five peptide fractions released from 3T3 cells. Extensive digestion of the fractions with pronase and trypsin produced a group of closely related glycopeptides in one chromatographic peak. The sugar chains were attached by an O-glycosidic bond to either serine, threonine or asparagine hydroxyl groups (Winzler et al 1967) Meezan/

Meezan et al 1969). Peptide fragments of trypsin-treated cell surface glycopeptides from erythrocytes are largely composed of serine and threonine (Winzler et al 1967). If trypsin acted on plasma membranes by slowly digesting away the membrane protein components leading to cell lysis many species of glycopeptide fragments should be obtainable. By producing few glycopeptide species from intact cells, trypsin may only be able to attack a limited number of the sensitive sites in PM proteins.

b) The use of agglutinins

Two classes of specific agglutinating proteins react with cells: plant lectins and immunoglobulins. Like enzyme molecules, large agglutinins may react at the cell surface or they might be able to penetrate the cell and react with their specific substrate internally. However, if they penetrate to a depth in the PM of more than half their own length, then it is unlikely or impossible that cells would be agglutinated by them. NANA molecules have been shown to be accessible at the cell surface since several cell types may be "hemagglutinated by myxovirus which specifically binds NANA. This operational definition of the cell surface by the use of myxovirus can be applied to agglutinins as well as to enzymes.

Electron microscopy of preparations of whole cell specific/

specific antibody reacted against isolated PM have shown that the antibody binds with only the outer PM leaflet (not the inner cytoplasmic leaflet) (Kite and Merchant 1961). The inner leaflet, although exposed to the antiserum, was apparently not antigenic. Therefore, even if antibodies do penetrate the PM, they would react with cytoplasmic antigens and not the inner PM leaflet which seems non-antigenic. Then the antibodies would be too deep to agglutinate cells. On this basis, it would seem that the agglutination of cells by antibody indicates that the antigen is located in the outer leaflet of the PM, i.e. the cell surface.

Kite and Merchant (1961) also presented evidence which indicated that virtually all of the antigenic cell surface material was sugar in nature with negligible contributions from lipid or protein components.

BHK-21/C13, and 3T3 cells, which have been transformed by SV40 or polyoma virus, and chemically transformed L1210 cells are specifically agglutinated by the lectins wheatgerm agglutinin and concavalin-A, (Burger and Goldberg 1967; Burger 1968; Inbar and Sachs 1969 a,b). The action of wheat-germ agglutinin can be specifically and reversibly inhibited by Nacetyl/

Nacetyl-glucosamine, Nacetyl-chitobiose and ovomucoid which has been interpreted by Burger and Goldberg (1967) to mean that Nacetyl-glucosamine is the primary wheatgerm lectin binding residue on the cell surface. A preparation containing a particle capable of binding the lectin was released from the cell by hypotonic shock treatment. The particle contained Nacetyl-glucosamine, and Nacetyl-galactosamine (Burger 1968).

Treatments of the cells with sodium periodate and NANase prevented agglutination by the wheatgerm lectin and this was interpreted to mean that NANA may have a structural relationship with the binding site (Burger 1969).

The effect of the lectins on untransformed cells is to be discussed later.

Mehrishi and Grassetti (1969) have used an inorganic reagent, 6,6' - dithiodinicotinic acid, to react specifically with sulphhydryl groups on the cell surface. The authors believe the molecule is unable to penetrate the cellular PM. Therefore they suggest that the surfaces of a tumor cell, lymphocytes and platelets, may contain sulphydryl groups.

c) Electrophoretic evidence of cell surface components

Charged groups exposed at the cell surface, whether they are components of the PM or are adsorbed from the medium, will give rise to an electrostatic field about the cell. By measuring the velocity of the suspended cell in a potential gradient/

gradient the ζ (zeta) potential and hence the cell surface potential may be ascertained.

Curtis (1967) has criticised the application of electrophoretic technique to cells on the following grounds. In order to use electrophoresis, it has been assumed that the surface charges are uniform over the area of the surface and that the cell-medium interface is impenetrable to counter ions. Neither of these assumptions is valid. Haydon (1961) and Seaman and Heard (1960) have suggested that the cell surface may exist as a matrix in depth which may freely allow access to counter-ions.

Curtis (1967) also pointed out that virtually all electrophoretic studies were carried out in a medium of monovalent cations only. He suggests that the cellular surface potential may be completely different under physiological conditions.

Finally, it is assumed that cells undergoing electrophoresis have smooth, round surfaces. Goldman and Follett (1969) have shown that rounded BHK-21/C13 cells possess a large number of microvilli and surface distortions which may tend to reduce the apparent surface charge and/or increase the surface area and decrease estimates of surface charge density (Curtis/

(Curtis 1967). Hunter (1960) suggested that this may be the case for erythrocytes. Since it is the charge effective in producing the ζ potential at the electrokinetic shear plane which is measured and not the surface potential directly these discrepancies may seriously limit interpretation of electrophoretic evidence.

Attempts to discover the molecular nature of the charged groups at the cell surface have been carried out by cell electrophoresis at different pH values. Changes in the cell mobility-pH curve might then be related to the dissociation of particular acid or basic groups at the cell surface. Cook et al (1960, 1961) applied trypsin and Nanase to erythrocytes and found that the treatments greatly reduced their electrophoretic mobility. It was concluded that sialic acid made up most of the cells' net negative charge. All cells so far measured possess a net negative charge and N- or O,N-acetyl-neuraminic acid is thought to contribute a large portion of the charge on many other cell types: lymphocytes (Cook and Jacobson 1968, Ward and Ambrose 1969) and for numerous tumor cells (Simon-Reuss et al 1964, Vassar 1963, Weiss and Hauschka 1970). Forrester et al (1962) found that BHK-21/C13 cells owed 35% of their surface charge to NANA.

A/

A number of other ionic species have been found at the cell surface by electrophoresis. Several cell types display $-NH_3^+$ groups (Cook and Jacobson 1968, Ward and Ambrose 1969). Phosphate groups may exist on BHK cells (Forrester et al 1962). The β and γ carboxyl groups of aspartic and glutamic acid have been found by Cook and Jacobson (1968).

4) Cell surface structure

Previously in this discussion, it was mentioned that some cell surface components, particularly the sugar moieties of glycolipids, were inaccessible to specific enzymes. A similar situation occurs with the effectiveness of the lectins. It has been shown that transformed cells are agglutinated by these proteins, whereas normal cells are not (Burger and Goldberg 1967, Inbar and Sachs 1969a, b). Thus the lectins have been suggested to be specific for malignant cells.

However, immediately after treatment with trypsin, normal cells also bind and are agglutinated by the lectins (Burger and Goldberg 1967, Inbar and Sachs 1969b). Hakomori et al (1967) have isolated a glycolipid from BHK-21/C13 cells which inhibits the agglutination reaction and which contains N-acetyl-glucosamine. It is considered by Inbar and Sachs(1969b) that/

that the cell surface lectin binding site is masked by protease sensitive materiel and in untransformed cells is inaccessible to the agglutinin.

Uhlenbruck et al (1968a, b) suggest that treatment of erythrocytes with trypsin or NANase reveals the formerly inaccessible glycolipids and Friedenreich antigens. The authors suggest that the negative surface potential, due in a large part to NANA groups, is capable of preventing the close approach of antibody molecules and that the removal of NANA enhances binding by lowering the surface charge.

By reason of its negative charge, NANA is also believed to mask antigens on the trophoblast and on neoplastic cells (Currie and Bagshawe 1967).

Kraemer (1967b) suggests that the configuration of the cell surface may alter during mitosis. He believes that the cellular electrophoretic mobility may change during mitosis but that the cell surface density of NANA remains constant. However, mitotic cells may have a different surface area than intermitotic (Goldman and Follett 1969) and Kraemer (1967b) had assumed the cells were smooth spheres.

5) Cell coats

Several workers have claimed that some normal and neoplastic cells are enveloped in a coat of acid mucopolysaccharide. Purdom et al (1958) and Forrester et al (1962), on the basis of electrophoretic studies, have suggested that malignant cells had/

had a greater negative surface charge than normal cells. Rambourg and Leblond (1967) and Defendi and Gasic (1963) found that cells were heavily stained at their periphery with acid mucopolysaccharide stains and they proposed that a thick intercellular coat of this substance existed on cells. Histocompatibility studies by Currie and Bagshawe (1967,1968), Currie et al 1968 and Sanford (1967) led these authors to propose that tumor cells displayed unusually large amounts of NANA on their surface.

These findings have since been held as a valid statement applicable to transformed cells in general. However, careful analysis by a number of workers not only casts doubt on these conclusions but suggest a completely opposite view, (Ohta et al 1968).

Analysis of BHK-21/C13 cells (Hakomori and Murakami 1968) of the isolated membranes of 3T3 cells (Wu et al, 1969) and of the virally transformed derivatives of these cells reveals that there is in fact, less NANA on all the membranes of transformed cells. A recent electrophoretic study by Weiss and Hauschka (1970) finds no correlation between malignancy, electrophoretic mobility and cell surface NANA. Thus transformed cells may, in fact, have a less negative cell surface./

surface. The fact that there was greater staining on transformed cells compared to normal cells and that the staining was sensitive to NANase but not to pepsin (Defendi and Gasic 1963) may be more consistent with the evidence that many trypsin resistent carbohydrate containing groups are masked on normal cells but exposed on transformed cells (Burger and Goldberg 1967).

At present, there seems little direct evidence supporting the existence of a distinct cell coat for most cell types. It is more likely that cell surface carbohydrate is part of the PM and therefore conclusions about the metabolism of the PM will apply to cell surface carbohydrate also.

III. THE MEASUREMENT OF CELLULAR ADHESION: INTRODUCTION

The methods most commonly applied to examine cell adhesion have been measurements of either the tenacity of the cell in maintaining previously established contacts or their ability to form new adhesions. With regard to the former, a number of techniques have been developed using force (hydrodynamic, centripetal, gravitational, or mechanical) to dislodge cells from cell-cell or cell-substrate adhesions.

Although both de-adhesion and the formation of new contacts occur *in vivo*, systems using de-adhesion have an important advantage over those which look at the *de novo* formation of adhesions. The latter usually requires manipulation of the cells and, especially, disaggregation of cells by techniques which may affect reaggregation. The techniques of de-adhesion differ in that they measure the strength of contacts formed *in vivo*, or at least after cells may have recovered from injury in handling. However, there are greater difficulties involved in interpreting de-adhesion.

A. De-adhesion studies

1) experimental methods

Coman (1944, 1961) and Brooks et al (1967) have attempted to estimate the force of intercellular adhesion by measuring the/

the force required to tease two adherent cells apart with glass needles. Weiss (1961) used a range of estimated shear forces to tear cells from a glass-substrate adhesion while Brooks et al (1967) used the same system to shear cells from aggregates in suspension. Others have studied cell adhesion to glass by allowing plated cells to be subjected to known forces directed so as to peel them from their substrate (Dan 1936, 1947; Berwick and Coman 1962; Easty et al 1960).

2) criticisms

De-adhesion techniques have several common disadvantages which may make it difficult to obtain a meaningful measure of cellular adhesion. Much of the force used to break cell contacts, both experimentally and physiologically (by cell locomotion), may go into overcoming the resistance of the cell to deformation. If the resistance to pulling or peeling the cell is substantial or if the strength of adhesion is greater than the tensile strength of the plasma membrane then the PM may rupture at the point of contact (Weiss and Coombs 1963, Brooks et al 1967). Muir (1967) found that when heart cells separated from one another, they displayed areas where either the PM had been lost or where a portion of the PM of another cell remained attached. In the latter case, the two membranes formed/

formed a "tight junction" and it was concluded that the attached PM had been pulled from a cell leaving the observed gaps.

A further criticism of some of the studies where a "force of adhesion" was measured has been made by Steinberg (1964, 1970) who argued that only the total energy involved in breaking a cell contact was important. The measurement of the energy of adhesion would require integration of the force over the distence it was applied rather than only the maximum force required to produce disaggregation.

B. Adhesion systems

When cells in a population are given an opportunity to contact each other, the probability of encounter resulting in adhesions may be taken as an expression of cellular adhesiveness. Two main techniques have been used to bring about cellular contact: (i) substrate mediated systems where intercellular collisions are caused by fibroblastic or amoeboid locomotion - migration systems, and (ii) suspension systems where dispersions of single cells are agitated to induce intercellular collisions. Cells have also been aggregated by centrifuging them into a pellet (Trinkaus and Lentz 1964).

1)/

1) migration system

In this system suspensions of single cells are plated out onto a surface (e.g. a petri dish). The nature of the adhesive interaction developed between cells faced with this situation *in vitro* may be identical with that *in vivo* since cellular contact interactions occurring in metazoan tissues display analogous cell-cell and cell-substrate relationships (Abercrombie 1966).

The best known example of a naturally occurring migration system is that of the cellular slime molds. Raper and Thom (1941) have described the transformation of a population from one of dispersed amoeba to that of a multicellular slug. During the dispersed state chance intercellular contacts are transient and unstable and aggregation is not detectable. However, after exhaustion of the food supply the intercellular encounters occurring as a result of chemotaxis result in stable adhesions and hence the formation of large aggregates of cells.

Aggregation resulting from cell locomotion has been observed with numerous other cell types, including sponge cells, which aggregate to form complete sponges,(Wilson 1907), species of soil amoeba (Band and Mohlok 1969) mammalian cells/

cells (Moskowitz 1963) and chick embryonic cells (Moscona and Moscona 1952). Weiss and Taylor (1960) aggregated cells on chick chorioallantoic membrane.

The aggregation of the cells into clumps on the surface of the culture dish is thought to be a measure of cellular ability to form adhesions and it has been suggested that "adhesive" cells should prefer (or be unable to break) mutual adhesions since these should be stronger than cell-substrate adhesions (Moscona and Moscona 1952).

Three important assumptions must be satisfied by the locomotion systems if valid conclusions about cell adhesion can be drawn from their use. The first is that the cells are freely motile so that the rate of aggregation is a direct function of cell adhesiveness. Galtsoff (1925) has suggested the possibility that cells which do not form aggregates, and are therefore judged non-adhesive, may in fact be relatively non-motile. Band and Mohrlock (1969) found that amoeba on glass did not aggregate at low temperatures and they concluded that adhesion was temperature dependent. However, it is more likely that cell locomotion is the temperature dependent factor. In other cases, the motions of some cell types may be governed by taxis as can be seen for the Dictyostelium species/

species (Bonner 1947). These cells avoid one another by negative chemotaxis during their vegetative cycle but develop positive chemotaxis.before slug formation (Gerisch 1968). The direction of cell locomotion may also be determined by the surface structure of the substrate, (e.g. contact guidance, Weiss 1934), (Curtis and Varde 1964). Abercrombie and Heaysman (1957) have shown that intercellular encounters may lead to changes in the direction of locomotion and they have termed this phenomenon contact inhibition of locomotion. Therefore it can be seen that other factors (including possibly the nature of the substrate, the physiology of the cell, taxis, and the sensitivity of locomotion to cell contact and temperature) may determine the frequency of intercellular contact and thus the apparent adhesiveness of the cells.

A second assumption is that "adhesive" cells will be trapped into aggregates because they prefer cell-cell contacts rather than those of cell-substrate. However, in cultures of fibroblasts, the degree of association between cells may be explained on the basis of the cellular control of locomotion, i.e. contact inhibition of locomotion (Abercrombie and Ambrose 1962), or rather contact control of movement. In cell monolayers/

monolayers one fibroblast encountering another is inhibited from approaching further once contact is made. The cells are able to move away from one another in any direction where intercellular contact is lacking (Abercrombie and Heaysman 1966). Cells which are not contact inhibited will not avoid moving over or under other cells. Conversely, cells in association may not move away from one another since movement not involving cell-cell contact may be inhibited (e.g. contact promotion, Curtis 1967).

A final assumption is that strong cell adhesion on a substrate expresses itself by aggregation of cells into a three-dimensional clump. As mentioned above, the degree of overlapping of cells may be a function of the control of locomotion rather than simply cell adhesion. The example of epithelia may show that these cells, which are adhesive to one another and which aggregate on glass and in vivo do not pile up but display strong contact inhibition and remain as a monolayer. Middleton (1969) found that the cells on the periphery of aggregates were unable to break away even through their own locomotion severely strained their intercellular contacts. Nevertheless, he found virtually no overlapping.

2) /

2) suspension systems

a) theoretical models of particle interactions

Two particles, such as cells, in suspension, may approach one another by the effects of (i) Brownian motion, (ii) hydrodynamic shear or (iii) mutual attractive force. The most thoroughly investigated situations have been those of lyophobic colloids where Brownian motion is responsible for interparticle collisions (i.e. perikinetic flocculation). Overbeek (1952) has reviewed both cases of perikinetic flocculation whose quantitative treatment has been developed by Von Smoluchowski (fast coagulation) and by Fuchs (slow coagulation). In both situations, the rate of aggregation of the particles is a direct measure of their adhesiveness (Overbeek 1952),

With particles the size of animal cells, few collisions would result from Brownian motion compared with those occurring if the suspension was agitated (Tuorila 1927, Curtis 1969). In order to account for the effect of shear on the particle collision rate, Von Smoluchowski has developed a new relationship where the collision rate (b) is directly proportional to the shear rate;

$$b_{ij} = {}^{4}/_{3}\, G n_i\, n_j\, (r_i + r_j)^3 \qquad \text{(eq.1)}$$

where G is the shear rate,

n/

n the concentration of particles i and j, and

r the radius of the particles.

Collisions mediated by shear may be thought of as occurring when a cell moving in a slipstream is overtaken by another cell in a slightly faster slipstream. As the distance of their separation approaches a small valve, at some angle of contact, their subsequent behaviour may be increasingly governed by the attractive and repulsive forces between their surfaces. Even if the cells are attractive the force of shear or of Brownian motion may break a contact once it is formed.

Curtis and Hocking (1970 developed a treatment for the aggregation of lyophobic colloids in an orthokinetic (e.g. shear) system.based on Swift and Friedlanders (1964) integration of Von Smoluchowski's collision rate relationship (eq. 1) for different particle sizes. By measuring the total particle concentration,

$$\ln(N_{\infty t} / N_{\infty 0}) = \frac{-4}{\pi} \phi E t K \qquad \text{(eq. 2)}$$

where N_∞ is the total particle concentration at time

t (0 or t),

ϕ the volume fraction of the suspended particles,

K the shear rate, and

E the collision efficiency.

The/

The shear in the system is laminar, being produced by the use of the couette viscometer (which is described later). Particle adhesion is measured as the collision efficiency of aggregation ($\dot{E}$). If certain assumptions are made about the nature of the particle interaction, then E may be a direct measure of the energy of the adhesion (Curtis 1969).

Some methods of measuring cellular adhesion as a function of the capability of the cell to form (stable) contacts may measure an adhesion different or perhaps insignificant compared with that of the final state of the contact. Two possible phenomena, among others, may operate to alter cell adhesion after contact is established:

1) Steinberg (1970) has suggested that the energy applied to bring two cells together may go into producing a high energy state (activation energy) which is necessary before cell adhesion (at a lower energy) can result. This example is analogous with an endothermic reaction. Therefore on a first assumption, the energy which may be measured and thought to be of cell adhesion may in fact be that of the "activation energy".

2) The nature of the cell surface and perhaps that of the adhesion, may alter after contact. For example, the permeability/

permeability of the PM increases after the formation of adhesive contact during the establishment of low resistance intercellular pathways (Loewenstein 1968). In response, the nature of the adhesive interaction at that point may also alter as a function of the new cell surface structure.

b) shear systems

Several methods of agitation have been used in order to induce collisions between cells in suspension.

The couette viscometer, described by Curtis (1969, 1970), is unique among these in that the value of shear is known and constant throughout the suspension. The cell suspension is introduced into a narrow space between two vertical, concentric cylinders and upon rotation of one cylinder about the other, laminar shear is developed. The danger of cell settling is remedied by the use of high viscosity media.

Several workers have used a system where the cell suspension is rapidly mixed by a stirrer revolving at several hundred r.p.m. in cuvettes or test tubes. The system has been used to measure the aggregation of platelets (Born 1962), chick embryonic cells (Jones, 1966, Kemp et al 1967) and slime mold cells (Born and Garrod 1968).

Slime/

Slime mold cells have been aggregated by placing the suspension in a rolling test tube (Gerisch 1960). The adhesion of a variety of cell types has been investigated by the use of gyratory shaking which was applied to chick embryonic cells by Moscona (1961), Garber (1963), Roth and Weston (1967) and Orr and Roseman (1969). Ede and Agerbak (1968) studied the aggregation of normal and mutant (talpid) chick embryonic cells by this method. The aggregation of sponge cells by gyratory shaking has been studied by Humphreys (1963).

Reciprocating motion has been used to investigate the aggregation of chick embryonic cells (Curtis and Greaves 1965, Roth and Weston 1967) and BHK-21/C-13 cells (Edwards and Campbell 1971a) and transformed C-13 cells (Edwards and Campbell 1971b).

c) criticisms of the methods

Two serious disadvantages of the gyratory shaker system have been discussed by Roth and Weston (1967) and Curtis (1970c) with regard to the measurement of adhesion by this method. The rotary motion of the flask sets up a velocity gradient in the suspension with a minimum at the vessel's center. This produces variable shear in the suspension and will tend to segregate cells and aggregates of different sizes into different areas in the flask. Therefore the collision rate/

rate will be a function of local particle concentration and the rate of aggregation will not be a direct measure of cellular adhesion.

The aggregation rate is more directly related to the collision efficiency in the reciprocating or couette system. Particles of all sizes are more likely to be distributed evenly in the cell suspension. However, variable shear is also a feature of the reciprocating system. The rate of collision (b) is a function of the shear rate, or rather the average shear rate; whereas the degradative effects of shear on aggregates (increasing as the square of their diameter) will be a function of the maximal value of shear in the system (Curtis 1970a).

3) assessment of cell aggregation in suspension systems

The degree of aggregation has been measured by simple qualitative microscopy of the cell suspension (Moscona 1961, Humphreys 1963, Gingell and Garrod 1969). Rigorous quantitation of aggregation was introduced by Curtis and Greaves (1965) using hemocytoØmetry which allowed the distribution of cells in aggregates and the kinetics of aggregation to be measured. Steinberg and Granger (1966) filtered the cell suspension onto micropore filters after various /

various incubation times and counted the distribution of cells in stained aggregates. Roth and Weston (1967) measured adhesion by counting the number of isotopically labelled cells collected by unlabelled aggregates using autoradiography of the aggregate sections.

Continuous recording turbidimetry has been applied to measure the aggregation of platelets (Born 1962), slime mold cells (Born and Garrod 1968), and chick embryonic cells (Kemp et al 1967). This method employs the rapid stirring of the cell suspension in a cuvette, as described previously, and records changes in aggregation as variations in the optical density of the suspension. Kemp (1970) has shown that there is a disparity between the results of the measurement of aggregation of chick embryonic cells by turbidimetry with that of hemocytometry which is a more direct method. Turbidimetry seems insensitive to the initial phase of cluster formation and cannot easily be related to the number of adhesions formed.

Electronic particle counters (Coulter counters) have been used to measure the aggregation of chick embryonic cells (Ball 1966, Orr and Roseman 1969), teratoma cells (Oppenheimer et al 1969) and BHK-21/C-13 cells (Edwards and Campbell 1971). This technique offers a particular advantage over the other counting/

counting methods used in that the error inherent in counting a small population sample is eliminated and the coefficient of variation approaches a value of 2% whereas in practise the limited size of the cell sample counted by hemocytometry alone produces an error about 3-fold greater (Miale 1962).

4) criteria of adhesiveness in suspension systems

a) aggregation rate

The rate of aggregation (or, more precisely, the collision efficiency) of cells in suspension is a direct measure of intercelluler adhesiveness. However, it is possible that the cellular aggregation measured in suspension does not accurately reflect the adhesive properties of the cell in vivo or in culture.

Curtis and Greaves (1965) examined the aggregation of chick embryonic cells by hemocytometry, counting the changes in concentration of single cells and aggregates of different sizes as aggregation progressed. The curve of single cell or aggregate density with time was fitted against that for fast, perikinetic flocculation developed by Von Smoluchowski (see Overbeek 1952) from which the rate of the aggregation could be derived. The measurement of aggregation was carried out by closely following the particle concentration during the early phase of aggregation during which time most of the adhesions/

adhesions had formed. In addition, only simple glucose-balenced salts medium was used. These conditions are presumably not likely to offer cells the opportunity to alter their surface by metabolism. Thus the "adhesiveness" of the cells at early times should remain unchenged during aggregation and the aggregation rate should be a function of only three parameters, i.e. the collision rate, the particle volume fraction, and the collision efficiency.

b) final extent of aggregation

Other studies have expressed aggregation as a direct relation of the relative size of the aggregates. Moscona (1965) has claimed that cells in suspensions which yield only small aggregates should be thought of as being non-adhesive. Aggregate size as the prime criterion of aggregation ability has been applied to measure adhesion of chick embryo cells (Moscona 1961, Garber 1963, Kemp 1968, Richmond et al 1968, and Glaeser et al 1968) and of sponge cells (Humphreys 1963). However, Ede and Agarbak (1968) claimed that cell "adhesiveness" was inversely related to final aggregate size. The use of aggregate size as a criterion of adhesion has been criticised by Curtis and Greaves (1965) and Lilien (1969) who suggested that other factors may be important in determining /

determining the size of cell aggregates.

c) factors affecting rate and extent

(i) The heterogeneity of the distribution of adhesive cell surface between cells in the population may be of prime importance in regard to the rate and extent of aggregation. Single cell suspensions from any animal tissue will be composed of several cell types. Even with cultured or cloned cells a proportion of the population may display different adhesive behaviour (e.g. mitotic cells, Tobey et al 1967). The demonstration of type specific intercellular adhesion (Roth and Weston 1967) may indicate that the interpretation of the results of aggregation of impure cell populations may be difficult. (See section on Mechanisms of Adhesion later).

(ii) Secondly, heterogeneity of the distribution of adhesive cell surface on individual cells will affect both the rate and, possibly, the extent of aggregation. It has been suggested that the adhesive behaviour of two cell types is a function of the non-random distribution of adhesive area on their surfaces: slime mold cells (Gerisch 1968) and BHK21/C-13 cells (Edwards and Campbell, 1971a, see later). It has been shown by these workers that the adhesive behaviour of these cells deviates from that expected of cells where all areas/

areas of the cell surface are equally "sticky".

We may think of a hypothetical situation where each cell has only one, small patch of adhesive surface. If one patch can stick only to another the rate of aggregation will be a function of the "adhesiveness" of the patches, the concentration of patches and the collision rate of patches. Aggregation will stop when each patch is involved in an adhesion. At this stage, the total particle count will have decreased by half (and will remain stable), and each cluster will contain two cells. As the size or number of patches increases, the aggregation rate will increase and the extent of aggregation will approach the completeness expected of cells with uniformly adhesive surfaces.

(iii) Curtis (1970a) has suggested that if the energy of intercellular adhesion is similar to the energy of the shear in the suspension then the larger aggregates may be degraded in size. The effect of the shear force will increase as the square of the diameter of the aggregate. Therefore, the extent of aggregate size may be limited by shear if the force is large enough. In this case, the final size of the clusters may reflect the energy of adhesion (Curtis 1969). However, the existence of an equilibrium as such must be demonstrated if/

if aggregate size is to be used in this way. This has not been done in experiments published so far.

d) aggregate shape

Ede and Agerbak (1969) have judged cellular adhesion by the shape of the aggregate. Cells from normal chick embryo limb bud form smooth, rounded clusters when aggregated. However, suspensions of talpid mutant chick embryo limb bud produced aggregates which were ragged. The latter were thought to be less adhesive by the authors. This relationship conforms to Steinbergs (1958, 1970) suggestion that cells will tend to maximize the number of their contacts, resulting in a lower energy configuration. The more adhesive the cell, the more contacts it will form. On the other hand, cellular mobility within the aggregate may also tend to promote more contact. Therefore, the shape of the aggregate may be governed by processes controlling cell movement (Abercrombie 1966). The collision efficiency of dead cells may be large, but their aggregate might be ragged. In addition, less adhesive cells may also produce smooth aggregates since they too will tend to a configuration of lowest energy.

A number of workers have claimed that the degree of cellular adhesiveness is related to the histological appearance of/

of the aggregate after one or two days in culture, (Moscona 1965, Lilien 1969). No evidence has ever been advanced in favour of this suggestion.

C. Disaggregation of Cells

With few exceptions, cells used for aggregation studies must be initially dispersed into a single cell suspension. Platelets (Born 1962) and slime mold cells (Gerisch 1960) are two examples which exist in dispersed states *in vivo*. Their adhesive behaviour *in vitro* has been shown to be qualitatively related to that *in vivo*. The use of cells from tissues or cell cultures necessitates their dispersal with unknown effects on subsequent reaggregation behaviour.

Gerisch (1968) has demonstrated that the adhesive behaviour of slime mold cells aggregated in suspension accurately reflects the adhesive behaviour displayed *in vivo* when the cells are cultured on glass. Non-aggregating mutants of *Dictyostelium* sps. and cells in the vegetative phase are non-adhesive in suspensions containing EDTA. Cells which aggregate *in vivo* also form clusters in suspension whether EDTA is present or not.

Begent and Born (1970) have shown that the aggregation of platelets *in vivo* requires ADP in the medium and that subsequently/

subsequently the platelets themselves release ADP and alter their morphology. The same requirements and events are associated with platelet aggregation in vitro, (see later).

The strength of intercellular adhesion and the existence of a meshwork of intercellular material are usually sufficient to prevent the easy dispersal of tissues or cell cultures. Mechanical force has been successfully used to dissociate sponge tissue (Wilson 1907) but chemical reagents are generally used to loosen cell contacts. Proteases (Willmer 1945), cation chelators (Zwilling 1954), high pH (Holtfreter 1943) and washes removing divalent cations (Herbst 1900, Humphreys 1963) alone or in combination have been most commonly used, usually with mechanical shear, to dissociate a tissue into a suspension of viable, single cells.

It should be asked why cells proceed to reaggregate once dispersed. The effect of the disaggregating agent may be relevant to an understanding of the mechanism of adhesion. However, a number of treatments causing cell dispersal may have unknown effects on cell surface adhesive molecules.
1) A tissue or cell culture may be dissociated by destroying intercellular material trapping the cells, (e.g. collagen).
2) The dissociation treatment may be detrimental to cell physiology and cause self retraction of cell processes engaged in/

in contact. The cells may be induced to move away from one another.

3) Attempts to separate the cells may lead to rupture of the cell surface at the points of adhesive contact. Weiss and Coombs (1963) have found that cells dispersed from glass substrates may leave cellular material behind on the glass which can be detected immunologically. In a thorough study of the affects of EDTA perfusion on the integrity of rat heart tissue, Muir (1967) found that the structural collapse of the tissue after perfusion led to rupture of the plasma membranes. Often opposite cells which had retracted from one another displayed small gaps in their PM. The other cell then usually displayed a five layered structure at its surface which Muir interpreted as its own PM and that of the opposite cell joined in a "tight junction" (which was not weakened by the chelating reagent). Muir (1967) suggested that the strength of the junction was greater than that of the PM and therefore rupture followed. The presence of a fragment of broken PM on the cell surface alters its composition and might conceivably affect subsequent cell adhesion.

4) Cell disaggregation might follow the extraction of a possible intercellular ligand (e.g. like an antibody) which agglutinates the cells.

Reaggregation/

Reaggregation could result if this intermediate is replaced. Humphreys (1963) has claimed that the dissociation of sponge tissue follows after the removal of an intercellular glycoprotein and calcium, and that when these factors are replaced in the medium the cells can reaggregate.

5) Alternately a dissociation procedure which uses an enzyme may result in the complete digestion of adhesive components on the cell surface. From a first approximation, further cell aggregation should be completely inhibited. If not, then any subsequent cell aggregation could be explained by one of three hypothetical mechanisms. (i) The destruction of the cell surface adhesive material may be only partial. Reaggregation *in vitro* might then be a function of the same components which caused adhesion *in vivo* and adhesive behaviour will be at least qualitatively similar in both cases. (ii) If the primary mechanism of adhesion *in vivo* is destroyed, subsequent cell aggregation may be a function of a secondary mechanism of adhesion. This mechanism may have been pre-existing or it could conceivably be an artefact generated by the dissociation treatment. Cellular adhesive behaviour *in vitro* might then be quantitatively similar to behaviour *in vivo* and yet be qualitatively distinct. (iii) During reaggregation/

reaggregation the qualitative or quantitative nature of cell adhesion may alter. Curtis (1961) has suggested a timing hypothesis where temporal changes in the contact behaviour of cells might result from cell interaction or changes in cell physiology.

Oppenheimer et al (1969) have shown that adhesion increases in a cell suspension which is synthesizing and incorporating amino sugars. Roth (1968) found that freshly dispersed chick embryonic cells altered from being non-specifically adhesive to specifically adhesive over a period of several hours in culture. Curtis (1970b) has also found that the collision efficiency of the cells of two chick embryonic tissue types altered during long periods in culture.

IV. The Morphology and Function of Cell Junctions

Four morphological classes of cell contacts have been identified using electron microscopy: zonula occludens, small gap junctions; zonula adherens, large gap junctions, and macula adherens (Fawcett 1966, Revel and Karnovsky 1967). These junctions have been found between cells in vivo and between cultured cells but different cell types may not be capable of establishing all of these junctions. A few cases of intercellular cytoplasmic bridging have been found but in the majority of cell types, bridging is not detectable (Fawcett 1961).

A. The zonula occludens

The zonula occludens (ZO) apparently functions as an impermeable seal between cells giving a layer of tissue the property of a fluid barrier (Farquhar and Palade 1963, Brightman 1967, Reese and Karnovsky 1967). The junction itself is characterized by the apparent narrow line fusion of the outer leaflets of the plasma membranes of opposed cells, probably forming a ring of junction around each cell (Farquhar and Palade 1963, Goodenough and Revel 1970).

Treatment of tissue with desoxycholate or not less then/

than 60% acetone renders the ZO permeable to lanthanum yet does not alter its appearance (Goodenough and Revel 1970). The junction is resistant to disruption by treatment with chelators of divalent cations (e.g. EDTA) (Benedetti and Emmelot 1967, Sedar and Forte 1964, Goodenough and Revel 1970) or by urea (Goodenough and Revel 1970).

There is some specialization of the cytoplasm adjacent to the ZO but no difference between the appearance of the plasma membrane at the ZO and elsewhere on the cell (Farquhar and Palade 1963, Goodenough and Revel 1970). Staining reactions thought to be specific for acid mucopolysaccharides are positive over most of the cell surface but are negative at the ZO (Rombourg and Leblond 1967, Benedetti and Emmelot 1967). The authors concluded that glycoproteins were absent from the cell surface at this junction. However, their results may also be explained by failure of the stain to penetrate the intercellular region.

The strength of intercellular adhesion at the ZO may be considerable since the junction remains intact and the adjacent PM is ruptured when cells are forced apart (Furshpan and Potter 1968).

B. Small-gap junctions

The term "tight junction" has formerly been applied to both/

both ZO and a recently recognised class of contact, the small-gap junction (GJ) (after Farquhar and Potter 1968, also nexus, McNutt and Weinstein 1970; macula occludens, Fawcett 1966). Revel and Karnovsky (1967) suggested that the GJ should be considered distinct from the ZO for several reasons. Rather than forming a belt around the cell the GJ appears as discreet patches of contact on the cell surface. Also the intercellular space of the GJ is permeable to colloidal lanthanum which indicates an intercellular separation of at least 20Å at the junction (Revel and Karnovsky, 1967, Goodenough and Revel 1970). Uehara and Burnstock (1970) have also demonstrated the gap by electron microscopy in sections of smooth muscle. Tangential and perpendicular sections and freeze-cleaved preparations of cells reveal that areas of GJ consist of hexagonal patterns of 70 - 75Å subunits about 10 - 20Å apart (Goodenough and Revel 1970, Deamer et al 1970). Negative stained preparations of isolated PM show areas of hexagonal patterns in patches on the membrane (Robertson 1963, Benedetti and Emmelot 1968).

Goodenough and Revel (1970) have also differentiated the ZO and the GJ on a chemical basis. Cells of mouse liver, which were fixed with glutaraldehyde and subsequently treated with/

with not less than 60% acetone, lost the appearance of the $\sim$ 20Å gap at the junction; yet the total distance across the two membranes, measured from the cytoplasmic surfaces of the inner PM leaflets, remained unchanged at 140 - 150Å. The sub-unit appearance of the junction was destroyed by acetone but the trilayer appearance of the PM was unaltered. Fixation with Os O_4, rather than glutaraldehyde, preserved the 20Å gap.

Desoxycholate had little or not effect on gap morphology. Goodenough and Revel (1970) have suggested that PM lipid may be important in maintaining the gap structure.

The GJ has been found to be completely unaffected by treatment with EDTA and conditions of divalent cation depletion (Muir 1967, Goodenough and Revel 1970). Muir (1967) also found that this junction was resistant to mechanical force and that the PM yielded before the junction was destroyed.

The GJ is considered to be a prime site of electrical coupling between cells (Loewenstein 1968, Goodenough and Revel 1970, Furshpan and Potter 1968). Coupling has been observed to occur between a wide variety of cells in vivo and in tissue culture, including early embryonic cells of the chick (Sheridan 1968), fibroblasts such as BHK-21/C13, transformed and malignant cells (e.g. polyoma virus transformed BHK cells, Py Y)(Sheridan 1970, Furshpan and Potter 1968). However, malignant /

malignant cells are not invariably capable of communicating since Jamakosmanović and Loewenstein (1968a,b) have found that neoplastic thyroid tissue was unable to form low resistance intercellular pathways. Molecules as large as fluorescein may also be passed between cells when low resistance junctions are present. This has been shown with a number of cell types, including BHK-21/C13, using iontophoresis (Furshpan and Potter 1968).

Flaxman et al (1969) attempted to correlate the onset of contact inhibition of cell locomotion with the formation of GJ using cultured BHK-21/C13 fibroblasts. Immediately after one cell was seen to collide with another contact inhibition developed, lamellipodial activity ceased and the cells began to retract from one another. Electron microscopic observations of sections of these cells showed that in a few cases, GJ could be found within three to ten minutes of the initial contact. An extensive 100 - 200Å gap (see later) existed over most of the contact.

Loewenstein (1966) also found that electrical contact between sponge cells developed rapidly after the formation of adhesive contact. Dissociated cells from Microciona or Haliclona sps. were manipulated into contact and electrical coupling was evident within 15 minutes. No special area of the /

the cell surface was necessary for coupling since the areas forming contact were randomly selected. No adhesion or coupling developed if the cells were kept at 5°C unless the aggregation promoting factor (of Humphreys 1963) was restored to the medium (see section on mechanisms of adhesion following).

From these results, Loewenstein (1968) has suggested that the presence of a specific cell surface factor was required before intercellular adhesion and subsequent electrical coupling could develop.

Loewenstein further proposed that the factor may function on the cell surface by constituting an electrically insulating seal between the space of the junction and the medium (Loewenstein 1968). Such insulation must exist since there is little shunting of current from the junction to the extracellular medium. Loewenstein (1968) suggests that the formation of the insulated compartment also has the effect of exposing the junctional surface of the cells to the low intracellular level of calcium concentration. It is also suggested that the loss of calcium from the junctional region promotes a local change in the structure of the PM leading to the formation of a low resistance intercellular pathway.

Direct intercellular contact may not be an absolute requirement for electrical coupling since Bennett and Trinkaus (1970) /

(1970) have shown that coupling exists across the blastula of Fundulus. However the contact is by way of the blastocoel which is insulated from the bulk medium.

The requirement for low calcium concentration in the coupling junction is consistent with the mechanical resistance of the GJ to conditions of calcium depletion induced by EDTA treatment which has been shown by Muir (1967), Benedetti and Emmelot (1968) and Goodenough and Revel (1970).

C. Macula adherens

The third contact specialization may be distinguished in sectioned material as a 200Å gap between opposite, parallel plasma membranes, with one or more thin, electron dense band(s) running the length of the gap, centrally positioned between the two cell surfaces (Fawcett 1966). The term desmosome is usually used to describe this structure seen as oval patches of 2500 - 4100Å diameter on cells (Tamarin and Sreebeny 1963) or as a band around the cell (Hama 1960). However, Farquhar and Palade (1963) suggested that desmosomes may also exist as a more extended structure and they have proposed the term macula adherens for the former.

The inner leaflet of each PM at the junction is coated by a thin plaque of electron dense material. In addition, bundles /

bundles of thin 40 - 60Å fibrils (tonofibrils) lead away from the plaque into the cytoplasm. The plaque, in thin cell sections, is not sensitive to trypsin treatment yet, surprisingly, pronase, pepsin and chymotrypsin digest away the electron dense material (Douglas et al 1970). Mukherjee and Williams (1968) found structural connections between the PM and the central band and they suggested that the desmosome area may contain more protein than the surrounding PM.

Invertabrate tissues display desmosome-like structures, septate desmosomes, which differ from those commonly seen in vertabrates. Septate desmosomes lack tonofibrils and the intercellular space contains septa bridging from one cell to the other (Locke 1965).

Bullivant and Loewenstein (1968) have suggested that the septate desmosome is the site of intercellular electrical coupling in invertabrate tissue. However, Goodenough and Revel (1970) have found that the GJ may also exist in the clam and may be the site of low resistance junctions. Unlike the ZO and GJ, the desmosome contacts are easily destroyed by treatment with EDTA, allowing the cells to separate (Muir 1967, Sedar and Forte 1964, Benedetti and Emmelot 1968). The morphology of the dense, intercellular bands /

bands is not affected by protease treatment (Douglas et al 1970). However, Rambourg and Leblond (1967) and Benedetti and Emmelot (1968) found that acidic groups, identified as NANA by the latter workers, were exposed on the cell surface at sites of opened desmosomes.

In spite of this intercellular material, Farquhar and Palade (1963) found that desmosomes were permeable to molecules of both hemaglobin and ferritin (100Å diameter). Early embryonic cells, such as in the chick, are thought to lack desmosomes (Overton 1962). However, Trinkaus and Lentz (1967) found examples of these junctions during gastrulation in Fundulus. Soto and Castejon (1969) have published the only example of which I am aware, of a desmosomal contact between two BHK-21/C13 cells.

Campbell (1967) has proposed that the bundled structure of the desmosomal tonofilaments develops by accumulation of disperse filaments into a plaque at the site of intercellular contact, possibly because of lateral movement of fibril attachment sites in the PM.

D. Zonula adherens

This junction, also known as the intermediate junction (IJ) or simple apposition (Furshpan and Potter 1968), is similar to the desmosome in that cells are separated by 100 - 200Å./

200Å. However, there are no specializations of the cytoplasm and no evidence of the existence of intercellular structure and this type of junction often exists over large areas of cell contacts (Fawcett 1966).

The 100 - 200Å gap is preserved whether or not the contact includes two flat surfaces or substrates with sharp projections normal to the cell surface (Cornell 1969a,b). Curtis (1967) has studied the relationship of fibroblasts to their glass substrate, using interference reflection microscopy. He has suggested that the points of closest approach (within resolution of the method) of the cells to the glass were no less than 100Å (see latter).

From these results, Curtis (1964) believed that there was no evidence for intercellular material at the IJ (i.e.that the material would have to be extensively hydrated or of very low density to escape detection). Farquhar and Palade (1963) found that the IJ was permeable to hemaglobin and ferritin. However, to conclude that no intercellular material exists by these criteria may be in error since the same authors found that the identical molecules permeated the intercellular, electron dense structure of the desmosomes.

Rambourg and Leblond (1967) found that the intercellular spaces of rat tissue were filled with stains specific for mucopolysaccharides. However, the precipitation of the stain may /

may have exaggerated the quantity of intercellular material believed to be present (see previously). Flaxman et al (1968) found that a 450Å gap existed between fibroblasts and their polymeric mitrocellulose substrate. The gap was filled with material nearly as electron dense as the cytoplasm yet it was absent from intercellular contacts and free cell surface.

Cornell (1969b) discussed the possibility that during dehydration for electron microscopy, any hydrated, intercellular polysaccharide may be particularly affected by shrinkage and would therefore not be subsequently observed.

On the other hand, the 100 - 200Å gap is probably real and not an artefact since it is often seen by electron microscopy together with each of the other classes of junction.

Muir (1967) and Sedar and Forte (1964) have found that the IJ is destroyed by the treatment of tissues with EDTA.

Each class of junction probably contributes to cellular adhesion since the ZO, GJ and desmosome have been shown to resist mechanical force. The fact that the plasma membranes participating in an intermediate junction are held parallel over considerable areas, and are not randomly folded, indicates that this junction also is adhesive. The existence of four morphologically, chemically and functionally distinct classes of junction is prima facie evidence that different mechanisms of adhesion operate. However, some may be more important in phenomena of morphogenesis and cell interactions than others.

V. Mechanisms of Cell Adhesion

A. Non-specific cellular adhesion

Cells are capable of adhering to a wide variety of substances *in vitro* (e.g. glass, plastic). *In vivo* cells adhere to their own type and other cell types and also to non-cellular substrates. Curtis (1960) suggested that the morphology and chemical nature of cell contacts displayed a similar pattern whether or not the contact was homotypic, heterotypic or between cell and substrate. On this basis, he proposed that cellular adhesive interactions depended on a mechanism of non-specific, long-range forces and suggested an analogy between cell adhesion and the interaction of lyophobic colloids. Derjaguin and Landau and Verwey and Overbeek (DLVO) (see Curtis 1960, 1967), developed a theory for the stability of lyophobic colloids where particle interaction is governed by two forces, one attractive and the other repulsive.

1) The DLVO theory

The attractive force is the London-Van der Waals dispersion force which declines as the inverse square of the distance of separation (Parsegian and Ninham 1970). Lyophobic particles are often prevented from making molecular contact because /

because of the existence of a second force of electrostatic repulsion due to their net negative charge. If this force is strong enough then the particles may adhere in the secondary (energy) minimum. In this configuration, the particle surfaces are separated by a small distance, held there in an energy minimum by a balance of the Van der Waals force and the electrostatic repulsive force which declines exponentially with increasing separation. There is no energy barrier to formation of the secondary minimum. Particles in this configuration may be redispersed relatively easily (Curtis 1966).

The electrostatic force will act as a barrier to closer approach of the surfaces. However, if sufficient energy is applied to push the particles together their surfaces may come close enough so that, once again the attractive energy predominates with the surfaces in molecular contact. This form of adhesion, known as the primary minimum, is essentially irreversible (see Curtis 1966, 1967).

2) <u>The DLVO theory applied to cell adhesion</u>

Curtis (1960) and Pethica (1961) have proposed that several features of colloid stability were similar to cellular adhesive behaviour. In particular, the morphology of the secondary minimum, where particles are seperated by a gap of 100-200Å, and the primary minimum, of less than 5Å, are analogous/

analogous with the zonula adherens and zonula occludens respectively. Also the fact that 100-200Å gap was found in both intercellular and cell-substrate adhesions, and that there is no evidence for the existence of material in the gap (Curtis 1964, and see previously) have been used as evidence that the DLVO theory is applicable to cellular adhesion (Curtis 1967). The theory predicts that cellular adhesion should be non-specific.

Several other factors have also been used to support this suggestion. Firstly, Parsegian and Ninham (1970) have recently recalculated the magnitude of the dispersed forces. Their results indicate that forces from each of the contributing components of the cell should sum to produce a total force of much larger energy with longer range than previously supposed (see Pethica 1961). Secondly, the effectiveness of chelating reagents and high pH in dissociating tissue has been explained by Curtis (1960) and Bangham and Pethica (1960), as due to the increase in the cells net negative surface charge. Reagents such as EDTA are thought to be effective by sequestering cationic counterions, and high pH by more fully ionizing the surface anionic groups. Holtfreter (1943) found that amphibian tissues disaggregated at high pH. Dan (1936, 1947) observed that echinoderm eggs formed weaker cell-glass adhesions /

adhesions at low cation concentrations and also at high pH. Herbst (1900) found that cell adhesion was weakened by low calcium concentration. Thirdly, it has been suggested that cellular contacts are more easily formed by the approach of narrow cell processes than by the apposition of flat cell surfaces since the narrow projection should meet less electrostatic resistance (Bangham and Pethica 1960). Band and Mohrlok (1969) and Lesseps (1963) have also found electron microscopic evidence of punctate intercellular contact.

Curtis and Greaves (1965) found that EDTA-disaggregated chick embryonic tissues reaggregated independently of temperature and of protein synthesis. The authors suggested that their results were consistent with the DLVO mechanism of adhesion since dispersal forces are unaffected by temperature changes in the biological range (however see later).

B. Specific adhesion

1) Sorting out

Many of the ideas about mechanisms of intercellular adhesion were developed from observations by Wilson (1907), Huxley (1911), Galtsoff (1925) and Holtfreter (1939) on the sorting out of mixed cell types. Sorting out was defined as the/

the rearrangement and segregation of each cell type within a mixed aggregate. The rearrangement was believed to proceed until the cells, moving by their own locomotion, reached a relative position which resembled that of the cell types *in vivo*.

Tyler (1947) and Weiss (1947) suggested that sorting out and positioning could be explained by specific cell adhesion. A number of workers have since attempted to measure cell adhesion by the relative positioning of cells in aggregates (Moscona 1962, 1965; Steinberg 1964, 1970).

2) *Differential adhesion*

Steinberg (1964) suggested that hetertypic as well as homotypic contacts must be adhesive otherwise there could be no explanation for the structural relationships between any two cell types in tissues and aggregates. Steinberg (1964,1970) therefore proposed that a concept of differential or selective adhesion could explain the results of sorting out. In this model, if the cells of one type, "a", adhere to themselves with greater strength than do those of another type, "b", (i.e. $a\text{-}a > b\text{-}b$). Then the strength of heterotypic adhesions should be intermediate, viz: $a\text{-}a > a\text{-}b > b\text{-}b$. Therefore, sorting out (and positioning) of motile cells could be explained on energetic grounds since all cell types cohere but "b" cells would/

would tend to be displaced by "a" cells from a-b and b-b adhesions.

Steinberg (1964) further suggested that differential adhesiveness between cell types might result from the number or arrangement of adhesive sites on the cell surfaces (i.e. the better and greater matching of sites resulting in greater strength of adhesion).

As evidence for sorting out and differential adhesion among cell types, Steinberg (1970) cited his work on the behaviour of mixtures of cells from different tissues of the chick embryo. A hierarchy of positioning was suggested to be a feature of the sorting out of these cell types with one cell type (a) always segregating to an internal position in an aggregate of two cell types (a and b). Another cell type (f) always segregated externally. Steinberg (1964) found that "a" segregated internally to all of the other cell types, "b" to all but "a", and "c" to all but "a" and "b", etc., e.g. $a < b < c < d < e < f$. The position finally taken up by one cell type was suggested to be a measure of its relative adhesiveness, with the more adhesive cells taking up internal positions.

3) Sorting out as a measure of specific adhesion

Several objections may be raised against the use of the sorting /

sorting out phenomenon as a measure of cellular adhesiveness, or as evidence of specific adhesion. First, Steinberg (1970) has postulated that both mutual adherence and relative motility of the cells are essential to the argument that segregation and positioning are functions of cellular adhesiveness. However, cells in mixed aggregates in suspension have never been shown to be motile.

Secondly, the timing of events during aggregation may be important in determining positioning and segregation. Curtis (1961,1970b) has put forward a timing hypothesis where cells may change their adhesive properties in time. If the timing is different between two cell types, one may aggregate first and be enveloped later by the second cell type giving the appearance of sorting out.

Thirdly, Curtis and Varde (1964) and Weston and Abercrombie (1967) have found that there is little displacement of individual cells in a 3-dimensional array possibly due to contact inhibition of locomotion. Weston and Abercrombie(1967) found that fused tissue fragments of different type occasionally tended to form aggregates with one tissue type fusing to and then enveloping the other giving the appearance of "sorting out".

If cells prove to be motile in aggregates the appearance of /

of sorting out could be adequately explained if a phenomenon such as specific contact inhibition or chemotaxis functions.

Finally, Steinberg (1964) has suggested, as explained above, that the concept of specific adhesion is not the only possible explanation of the role of adhesion in sorting out.

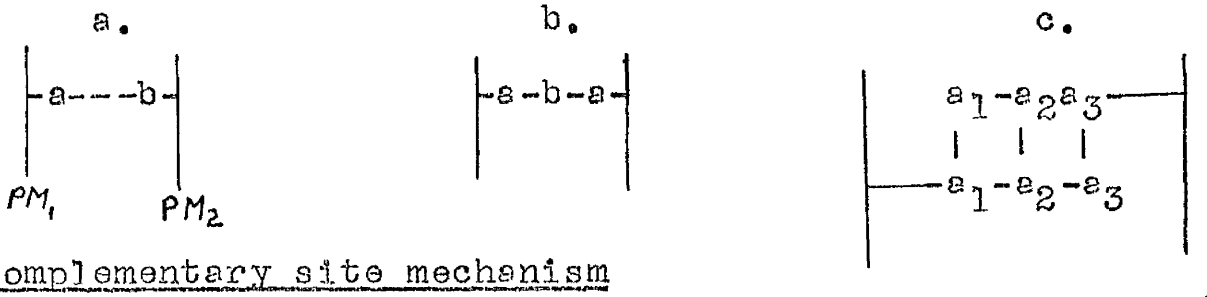
Curtis (1970 a,b,c) measured the collision efficiencies of mixtures of the cells of three different sponge species, and of mixtures of chick embryonic neural retinae and liver cells and has suggested that there is little evidence for specific adhesion among these cell types (see below however). Curtis found that, at most, about 10% of the cells might display specific adhesion, this being the error of the method. The technique used by Curtis (1969, 1970a), to measure specific adhesion offers the advantages that it measures adhesiveness directly by the collision efficiencies of the cell types and does not depend on identification (not always unequivocal) of the individual cell types (Curtis 1970a).

C. Mechanisms of specific adhesion

1) Models of cell surface interactions

Tyler (1946) and Weiss (1947), in addition to suggesting that cells adhered specifically, drew an analogy with the interactions of antibodies and antigens and proposed that cells could adhere by specific antigen and antibody-like molecules on /

on their surfaces. In all, there have been at least three models proposed for specific adhesion by cell surface molecular interaction and they each take one of the following forms. a) Complementary site mechanism: where one component on the surface of one cell specifically binds to a different component on another cell. b) Symmetrical sites: a "cement" or molecule is specifically bound to a site on each cell's surface, much like immune agglutination or hemagglutination. c) Roseman (1970) has suggested that identical (polysaccharide) components on the cell surface may bind intercellularly by hydrogen bonding.



2) Complementary site mechanism

a) Yeast mating strains

Although this mechanism was originally proposed by Tyler (1947) and Weiss (1947) to explain the behaviour of animal cells , the most unequivocal example of this (-a-b-) type of adhesion comes from interactions of the mating strains of the yeast Hansenula wingei. Brock (1965) and Taylor and Orton (1967) have found that the cell wall of each strain contains/

contains a glycoprotein which binds specifically to a different glycoprotein on the surface of the complementary gamete (Brock 1958, Taylor 1964). The glycoproteins have been isolated from the cell walls and have been found to be capable of inhibiting adhesion between complementary gametes (Crandall and Brock 1968).

b) Chick and mouse embryonic cells

(i) Evidence for specific adhesion

Strong evidence for the existence of specific or selective adhesion between chick or mouse cell types has been obtained by Roth and Weston (1967) and Roth (1968). In their experiments cells from various tissues of the embryo were labelled with ^{3}H-thymidine *in vivo* and then added to suspensions of tissue fragments or preformed aggregates in a growth medium. Cells of one type were added to aggregates of the same or different type. The number of labelled cells collected by the aggregates over a period of time was measured by counting cells in autoradiographs of sectioned aggregates. Their results showed that aggregates shaken with homotypic cells in reciprocal experiments collected more cells than those in heterotypic combinations.

This technique is the most direct of any used for the determination of specific adhesion since the only parameter measured is the number of cells collected by the aggregates.

The/

The authors also found that there were two phases of collection. In the first phase, non-specific aggregation was found between dispersed cells of chick liver and neural retina. During the second phase, after 4 or 5 hours, of incubation, non-specific cell-cell adhesion ceased. Some conditions of metabolic inhibition (e.g. low temperature or puromycin) reduced or prevented the onset of complete specific adhesion during the second phase.

The use of conditioned medium halted or reversed the effects of inhibition (see later) and promoted greater and earlier collection. On the basis of these findings, Roth (1968) suggested that two different types of adhesive components existed on the cell surface; one for specific adhesion and one for non-specific adhesion,

Curtis (1970b) has criticised the conclusions which Roth and Weston (1967) and Roth (1968) have drawn from their experiments and has suggested that since only small proportions of the total cells in suspension were collected by aggregates (Roth 1968), therefore specific adhesion was a property of only a small minority of the cell population. On the other hand, it may be that the dispersed cells would, because of their greater relative concentration, aggregate rapidly and extensively among themselves and not to pre-formed /

formed aggregates. Thus, pre-formed aggregates might make only a few adhesions to newly formed aggregates of labelled cells but the number of cells collected would be exaggerated by the number of labelled cells managing to aggregate and the proportion of specifically adherent cells would be over-estimated.

Curtis (1970b) has also suggested that the addition of fresh, trypsin-dispersed cell suspensions to suspensions of aggregates by Roth and Weston (1967) may also carry over enough trypsin to modify the adhesive properties of the aggregates' surface. However, Roth and Weston (1967) used a growth medium which contained embryo extract and its tryptic inhibitory properties must be considered (Wallis et al 1969).

(ii) Specific adhesion mechanism of chick embryonic retinal cells

Roseman (1970) proposed a novel mechanism for specific adhesion, involving an enzyme-substrate complex. This can perhaps be viewed as an example of a complementary site mechanism. Neural retinal cells were able to incorporate galactose, from externally added UDP-galactose, into macromolecular form at the cell surface. UDP-glucose did not compete, and the galactose incorporated could be recovered as galactose.

The incorporation was not merely by leaky cells since autoradiography /

autoradiography showed that over 90% of the cells became labelled if isotopic UDP-galactose was used. Cells would also catalyze the transfer of UDP-galactose to exogenous specific galactose acceptors. Thus the specific galactosyl transferase seems to exist on the cell surface and to effect reactions outside the cell.

Involvement of the enzyme in specific adhesion was suggested by the observation that galactose acceptors (of high and low molecular weight) inhibited the collection of neural retina cells by neural retina aggregates in the system described by Roth and Weston (1967).

With this evidence, Roseman (1970) has suggested that chick embryonic neural retina adhesion is due to the formation of an enzyme-substrate complex between the enzymes on one cell and galactose acceptors on the other cells surface. Such an acceptor might be an incomplete glycolipid or glycoprotein. The adhesion would be terminated by the presence of UDP-galactose (an example of "contact modification", see Hakomori 1970).

3) Symmetrical site models

In the examples cited below, evidence has been obtained which suggests that intercellular adhesion is a function of

a/

a cell surface "cement". The cement may be analogous to bivalent antibody which specifically agglutinates two cells. In some cases, specific divalent cations have been thought to act as adhesive "bridges" between cells. In order to invoke the symmetrical site model, it is necessary to identify both the cell surface component and the intercellular component of the adhesion. Specificity must reside in both. However, unlike antibody-antigen interactions, in no example investigated so far has the existence of a second component been proven.

(a) The platelets

The behaviour of platelets is one of few examples where aggregation *in vitro* can be shown to depend on the same mechanism which causes adhesion *in vivo* (Begent and Born 1970). In studies of most other cell types, the relationship between the two remains speculative.

Platelet adhesion is initiated by reaction with ADP in the medium. The first stage of this reaction involves morphological changes in platelet shape and their aggregation into loose clusters. Fibrinogen and calcium are both required for cluster formation and it is believed that they bind platelets together by ionic forces (Born and Cross 1963, Cross 1964, Born 1968).

(b) Sponge aggregation

(1)/

(i) Evidence for specific adhesion among sponge cells

Two kinds of observations have led to the suggestion that specific adhesion is important in sponge cell behaviour. Firstly, Wilson (1907) found that certain sponges, when dissociated and platedout on glass, reaggregated. If cells from more than one species were included, then after reaggregation, the cells sorted out and reconstituted small, complete sponges of each species. Secondly, Humphreys (1963) found that mixtures of cells of two species (Microciona and Heliclona) aggregated into clumps of either one species or the other as detected by the species specific colour of the aggregates.

Wilson's (1907) test for specificity can be criticised on the grounds that cell adhesion could be selective rather than specific and that the sorting out of cell types may perhaps be a result of other phenomena (e.g. contact inhibition, see previously).

Humphreys' (1963) interpretations were based on the results of experiments using gyratory agitation and measuring final aggregate size. The author has not ruled out the possibilities that these techniques introduced artefacts (e.g. the timing hypothesis, Curtis 1961, 1970b, combined with segregation of cells from aggregates in gyratory flasks, Roth and Weston 1967, see previously). In addition, Humphreys (1963)/

(1963) identified the cells by their species specific pigmentation. If all the cells are not coloured then a misleading indication of the amount of selective adhesion may result from observing only the cells in pigmented aggregates.

Curtis (1970a, c) measuring the intercellular collision efficiencies in interspecies mixtures, found no evidence of specific adhesion with these or other species (see previously).

(ii) The mechanism of sponge cell adhesion

Sponge cells which were mechanically dissociated from tissue reaggregated rapidly, extensively and independently of temperature, (Humphreys 1963). However, if the tissues were dissociated by washing in media free of divalent cations, subsequent aggregation followed a different time course. Small aggregates were produced initially but more extensive aggregation was dependent on temperature, long periods of incubation in culture and calcium or magnesium in the medium.

Aggregation (i.e. judged by final aggregate size) was enhanced by addition of the calcium and magnesium free medium in which the cells had been disaggregated. After this addition, aggregation was temperature independent (Humphreys 1963).

This led Humphreys (1963) to conclude that a factor, together with calcium in the medium caused cell adhesion by acting as an intercellular link or cement.

The/

The active washing extract was purified and examined by ultracentrifugation and Humphreys (1965) concluded that it contained a particulate glycoprotein. The factor probably originated from the cell surface during the washing procedure since Humphreys (1965) has shown that homogenized cells released/no detectable factor. The factor was heat labile and non-dialyzable but in further experiments the presence of the glycoprotein could not be correlated with aggregation-enhancing activity (Humphreys 1969).

Humphreys (1963) has shown that the factor operates species specifically. Extracts from one cell type will not enhance aggregation of any other cell type.

The effect of the factor on cell aggregation is not inconsistent with other mechanisms which might increase aggregate size. For example, it may function to increase aggregate size by enhancing cell physiology and the metabolism of cellular adhesive components. Humphreys (1963, 1969) believes the factor acts as a cement and sponge cell adhesion has been discussed as a model system in these terms (Lilien 1969). However, the connection between the factor and the symmetrical site model has not been rigorously established, nor has the function of calcium in this system. Although Humphreys (1963) has /

has suggested that calcium acts by specifically binding complementary groups between cells and factor (Humphreys 1963, 1970) has found that the activity of the factor is irreversibly destroyed in the complete absence of calcium. This may indicate that calcium is important in stabilizing the molecular structure of the factor (see later),

(c) The roles of calcium and conditioned medium

Roux (1894) and Herbst (1900) discovered that embryonic amphibian and sea urchin cells disaggregated when placed in media free of calcium and magnesium. Since then, other workers have found that embryonic avian and mammalian tissues are also more easily dispersed in media lacking divalent cations or after treatment with chelating reagents (e.g. EDTA) (Zwilling 1954, Curtis and Greaves 1965). (See discussion of Disaggregation previously).

For these reasons, cell adhesion has been thought to be dependent on calcium and magnesium. Several workers have suggested that calcium acts by bridging anionic molecules on the cell surfaces or between cells and intercellular cement (Coman 1954, Bangham and Pethica 1960, Steinberg 1958,1962). Calcium has been claimed to be more effective than other cations in this regard (Steinberg 1962).

Curtis /

Curtis (1962, 1966) has suggestedthat divalent cations function as counterions to the repulsive cell surface charge. This suggestion has been more fully reviewed above.

Steinberg (1962) attempted to identify the effect of calcium on cell adhesion and differentiate between the proposed roles in either counterion or ionic bonding. Steinberg (1962) measured the reaggregation of cells of EDTA dissociated amphibian tissue as a function of pH. The pH dependency of calcium binding to the cells was also measured by the use of radioactive calcium ^{45}. Steinberg (1962) found that re-aggregation was best at pH 6-10 and that Ca^{45} binding was maximal at pH 7-8. He interpreted his results to mean that anionic sites (carboxyl groups) were binding calcium and that calcium caused cells to adhere by bridging carboxyl groups on opposite cells.

Three criticisms may be levelled at Steinberg's conclusions.

i) Steinberg (1962) tested re-aggregation by the use of the migration system. Curtis (1963) has shown that no cellular locomotion is detectable at a pH lower than 4. It is probable that cell locomotion is pH dependent, and since aggregation in the migration system is a function of cell mobility, Steinberg's conclusion may be in error.

ii) /

ii) Steinberg measured the amount of cell bound calcium after exposing cells to the Ca^{45} containing solution for a few minutes, a length of time which he thought would eliminate the possibility of intracellular uptake of calcium. However, no evidence was presented to indicate that the bound calcium was limited to the cell surface and not to intracellular sites.
iii) The precise chemical formulation of Steinberg's proposed calcium-carboxyl group interaction is puzzling since the carboxyl groups most prominent on the cell surface, such as the neuraminic acids, have calcium binding constants which may be similar to that of acetate where K_1, for most methods, lies between $10^{0.53}$ and $10^{0.77}$ (Sillen and Martell 1964). In addition, the structure of the cell-calcium complex proposed is $-COO^{-}\ Ca^{++}\ \bar{O}OC-$ for which there appears to be no chemical precedent whatsoever.

The physiological effect of calcium depletion on cell adhesion has not been considered. In experiments where the formation of histotypic aggregates is used as the criterion of adhesiveness (e.g. Humphreys 1963) the lack of calcium during prolonged periods of incubation may have a deleterious effect on cell metabolism and the generation of adhesive cell surface.

Conditioned medium has been found to enhance cell aggregation (Roth 1968, Lilien 1969). Curtis and Greaves (1965) and /

and Taylor (1961) found that serum was capable of inhibiting cell adhesion. Curtis and Greaves (1965) isolated a protein component of horse serum which they suggested was destroyed by cell metabolism. If the protein was not destroyed (e.g. at low temperatures) then it inhibited aggregation. Orr and Roseman (1969) found that a protein derived from horse serum enhance chick embryonic neural retinal cell aggregation. Garber (1963) and Glaeser et al (1967) found that the presence of almost 30m Molar glucosamine inhibited chick cell aggregation probably due to the toxic properties of this amino sugar in high concentrations.

D. Other Cell surface adhesive components

Cells of some of the slime molds and of a mouse teratoma line have been recently shown to produce cell surface, sugar containing macromolecules may be required for adhesion and aggregation. In the case of the slime molds, these compounds have been shown to be species specific (Gerisch et al 1969).

(a) Aggregation of Dictyostelium discoides

In studies of slime mold aggregation, Gerisch (1968) found that the contacts of amoebs of the vegetative and slug-forming phases could be differentiated. Vegetative cells formed rounded aggregates in suspension but clumping was inhibited by addition of EDTA to the medium. On the other hand /

hand, slug-forming cells which aggregated on glass and also in suspension, produced clumps which were not sensitive to EDTA. However, the geometry of the cell contacts were altered by EDTA treatment. In suspension, these cells formed flat rather than round aggregates. Gerisch (1968) suggested that the EDTA resistant adhesion sites were distributed in a belt around the cell surface.

It was found that macromolecules containing fucose, mannose and Nacetyl-glucosamine could be extracted by the phenol procedure from both slug-forming and vegetative cells, but in greater amounts from the latter.

Immunochemical studies of intact cells indicated that both types displayed cell surface antigens identical with components of the phenol extract. Neither the isolated antigens or the cellular immunogenicity was influenced by pronase or α-amylase, however periodate treatment destroyed both (Malchow et al 1967, Gerisch et al 1969).

Immunoelectrophoresis of the phenol extract fractions showed several antigens, one of which was greatly increased in extracts from slug-forming cells, compared to vegetative cells, (Gerisch 1968, Gerisch et al 1969).

Univalent antibody fragments prepared against cell homogenates completely inhibited cell contact formation. The antibody /

antibody was shown to bind to the cell surface but periodate treatment of the cells prevented binding. Slug-forming cells bound at least 10-fold more antibody than vegetative cells (Beug et al).

(b) Teratoma aggregation

Specific cell surface sugar groups may also be important in the intercellular adhesion of a mouse teratoma cell line (Oppenheimer et al 1969). The cells of trypsin dissociated teratoma tumor aggregated slowly when incubated as a suspension in a complex medium but not at all when in a simple, glucose-balanced salts medium. A single component of the complex medium, L-glutamine, was shown to be responsible for this effect. L-glutamine could be replaced by the hexosamines glucosamine and mannosamine, but other sugars and their amino and N-acetyl derivatives were ineffective.

The effect of L-glutamine depended on metabolism and it was abolished by general metabolic inhibitors (e.g. fluoride) and also by the competitive inhibitors of L-glutamine metabolism 6-diazo-5-norleucine (Don) and O-diazoacetyl-serine (azaserine).

The authors have concluded that L-glutamine functions as a source of amino groups in the syntnesis of amino sugars which are thought to be part of the cell surface and required for cell adhesion.

(c) /

(c) Specific cell surface receptors

Some workers have attempted to draw conclusions about the existence of cell surface receptors by the effects on cell behaviour of particular chemicals added to the medium. Garber (1963) and Glaeser et al (1967) added high concentrations of glucosamine to the medium of chick embryonic cells (see above), and Cox and Gesner (1965, 1968) added a number of sugars to cells in culture and claimed that L-fucose was an important determinant of the cell surface in regard to cell behaviour. However none of the authors have attempted to discount the likely possibility that additions of high concentrations of these sugars to the medium may affect cell physiology and that subsequent alterations in cellular behaviour may be a non-specific consequence.

EXPERIMENTAL

I. General Methods

A. Introduction

1) BHK-21/C13 cell growth

The purpose of this study is to attempt to investigate the relationship between certain molecular components of the cell surface and adhesive behaviour. This requires the use of a cell type with a homogeneous population which is easy to manipulate and grow; a requirement best satisfied by a cell line.

The fibroblastic line BHK-21/C13 (C13) (Stoker and Macpherson 1964) was used throughout and fresh, low passage stocks were obtained from the Institute of Virology, Glasgow. The cells were grown on the surface of glass bottles (area, 120cm^2)[1] or, in some experiments, plastic bottles [2] (area 75cm^2).

The growth medium was enriched Eagles[3], 8 vols.; calf serum [4], 1 vol.; and tryptose phosphate broth [5](TPB), 1 vol. (811 medium after Stoker and Macpherson 1964). Once mixed, the medium deteriorated slowly, even at -20^{o}C, and therefore it was made up and used fresh on each occasion. The cultures were buffered with 5% CO_2 and 95% air[6] as the gas phase in the sealed bottles.

The /

[1] Superscript refers to Appendix I with details about materials.

The cells were seeded as a single cell suspension at approximately 2 x 10^4 cells/cm^2 and grown for two or three days at 37°C until the desired density had been reached. In most cases, this was at confluency (about 2 x 10^5 cells/cm^2) but pre-confluent cultures were also used on occasion (see fig. 1, 2). The growth and adhesive behaviour was subject to variation depending perhaps on the serum batch used and the passage history of the cells. Therefore, fresh cells were obtained once a month and were discarded after completing 15 or, occasionally, 30 passages.

2) Cell suspension

In order to produce a single-cell suspension for growth or aggregation purposes C-13 cultures were first drained of 811 and then gently washed three times with 10 ml. volumes of a divalent cation-free saline solution (tris[7]) at pH 7.4 and room temperature. A 10 ml. solution of 0.05% Difco trypsin-EDTA[8], at room temperature, was then washed over the cells and drained away, as the cells began to detach. The trypsin solution was routinely assayed by the method of Schwert and Takenaka (1955) and found to contain 250-300 BAEE units/ml. After two minutes of trypsin-EDTA incubation, ice cold tris was added to the culture and the bottle was briefly shaken to detach the cells. The suspension was then washed by centrifugation at 200g for 3 min. at 0°C.

(a) /

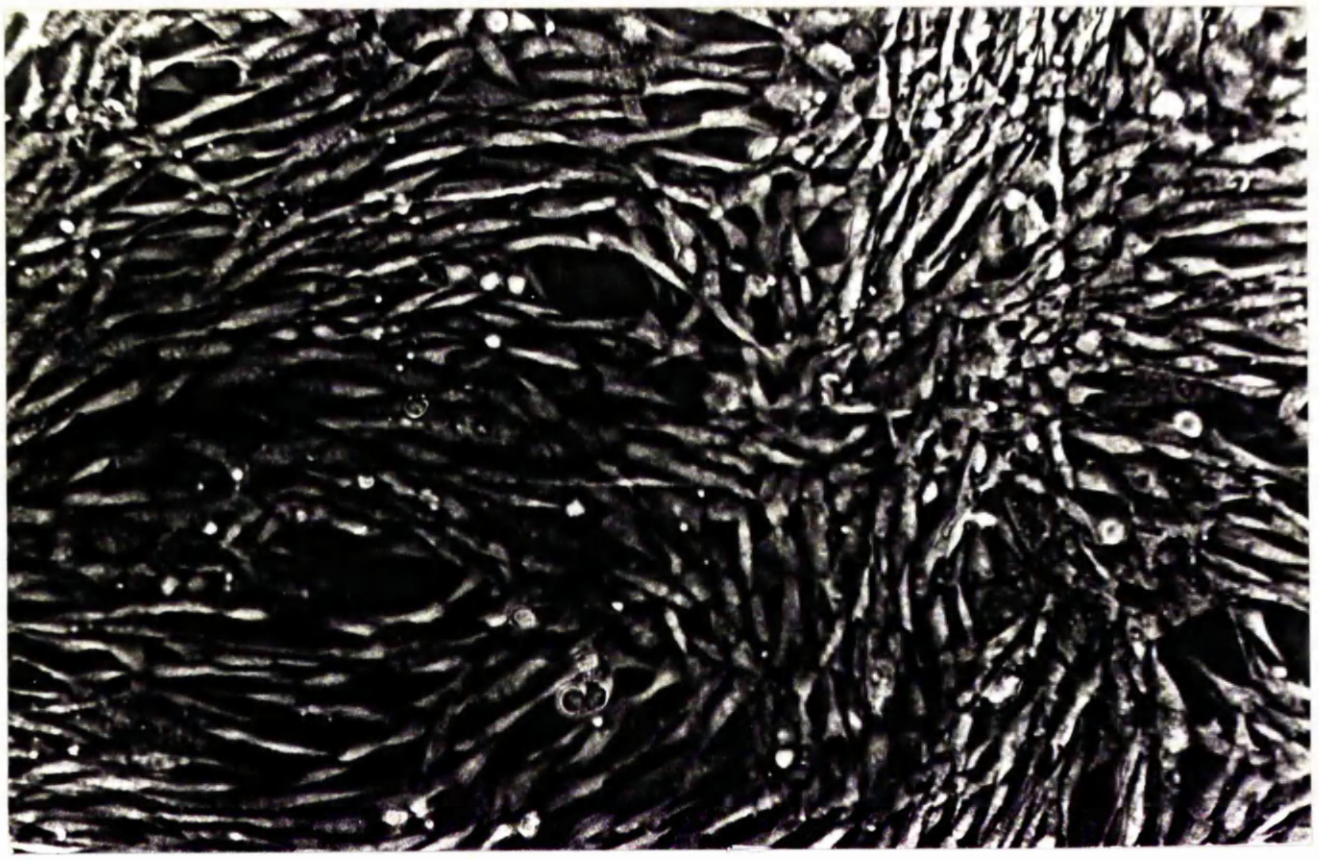


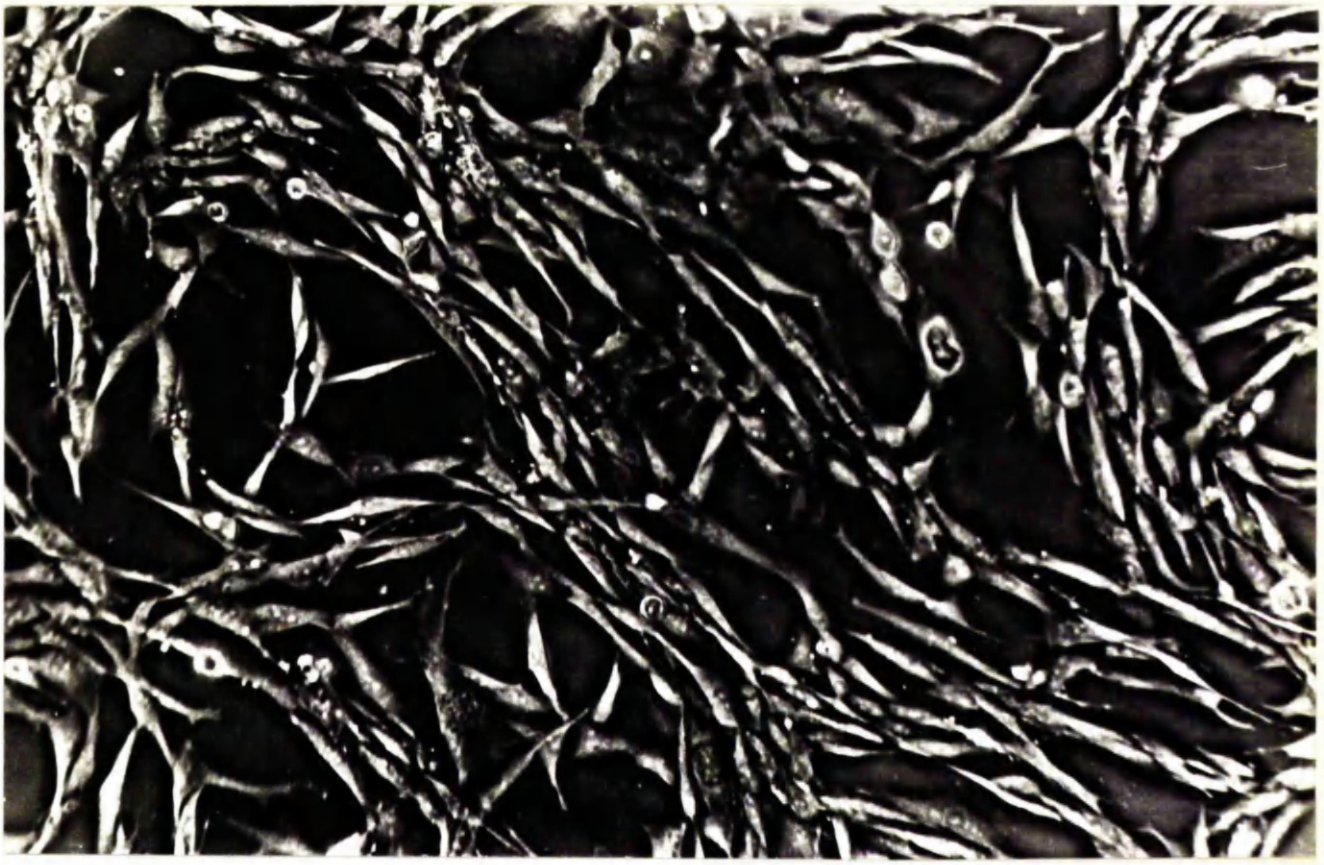
Figure 1.

"Confluent" culture of C-13 cells grown on a plastic surfase in 811 medium. Note the regimented alignment.

Phase optics,

Magnification: x675

a.



b.

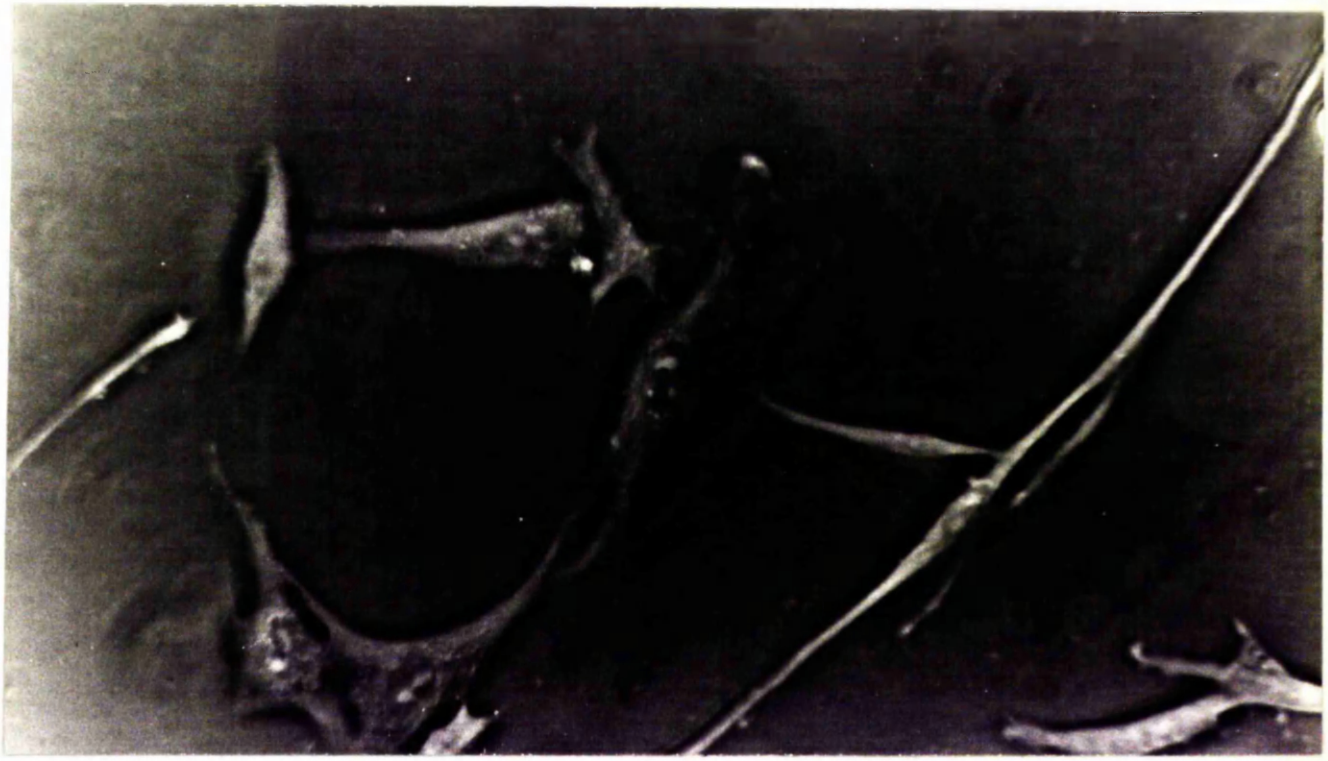


Figure 2. Low growth density cultures. In 2a) notice the develop -ment of alignment and the proportion of mitotic cells (arrow).

Phase optics,

Magnification: x675 (2a), x2700 (2b)

(a) Growth

For growth purposes the pellet from the first washing was re-suspended in tris at 0°C, by gently flushing with a pasteur pipet to break up the remaining aggregates. The cells were then washed once more in tris and then re-suspended in 811 at 0°C before being plated out.

(b) Aggregation

For aggregation purposes, the initial pellet was washed twice by centrifugation in ice cold tris and once more in Hanks solution [9]. Finally, the cells were re-suspended at about 10^6/ml. in Hanks and kept at 0°C before use within one hour. The low temperature was used to prevent aggregation, (Edwards and Campbell 1971a).

A suspension produced by this method contained, in addition to single cells, a very low proportion of two or three cell aggregates. By phase microscopy the cells were rounded and refractile. A small fraction ($\sim$ 1-10%) were neither rounded nor refractile and took up trypan blue. After some time at 37°C incubation, these cells tended to disappear probably by lysis (see figs. 3, 4).

The trypsin-EDTA procedure functioned adequately until used on old, multilayered cultures. Here a great amount of mechanical force was necessary to suspend the cells but a greater proportion were injured and therefore experiments were always /

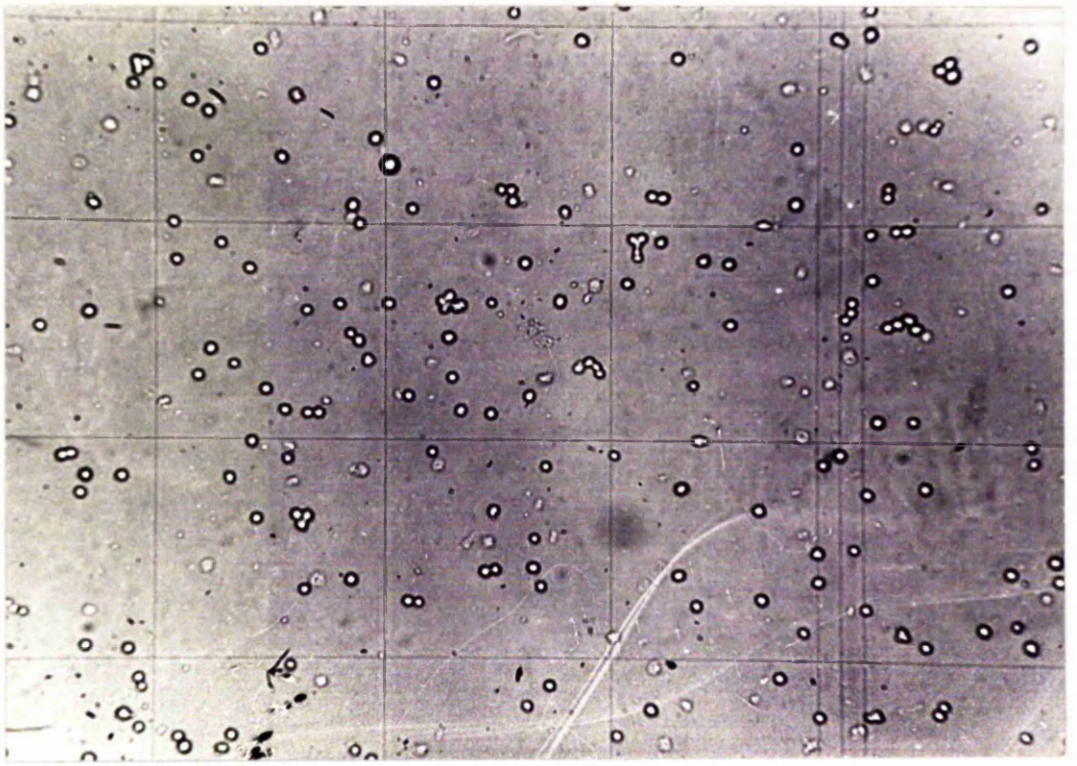


Figure 3.

An initial single-cell suspension also containing a few residual clusters.

Brightfield illumination,

Magnification: x284

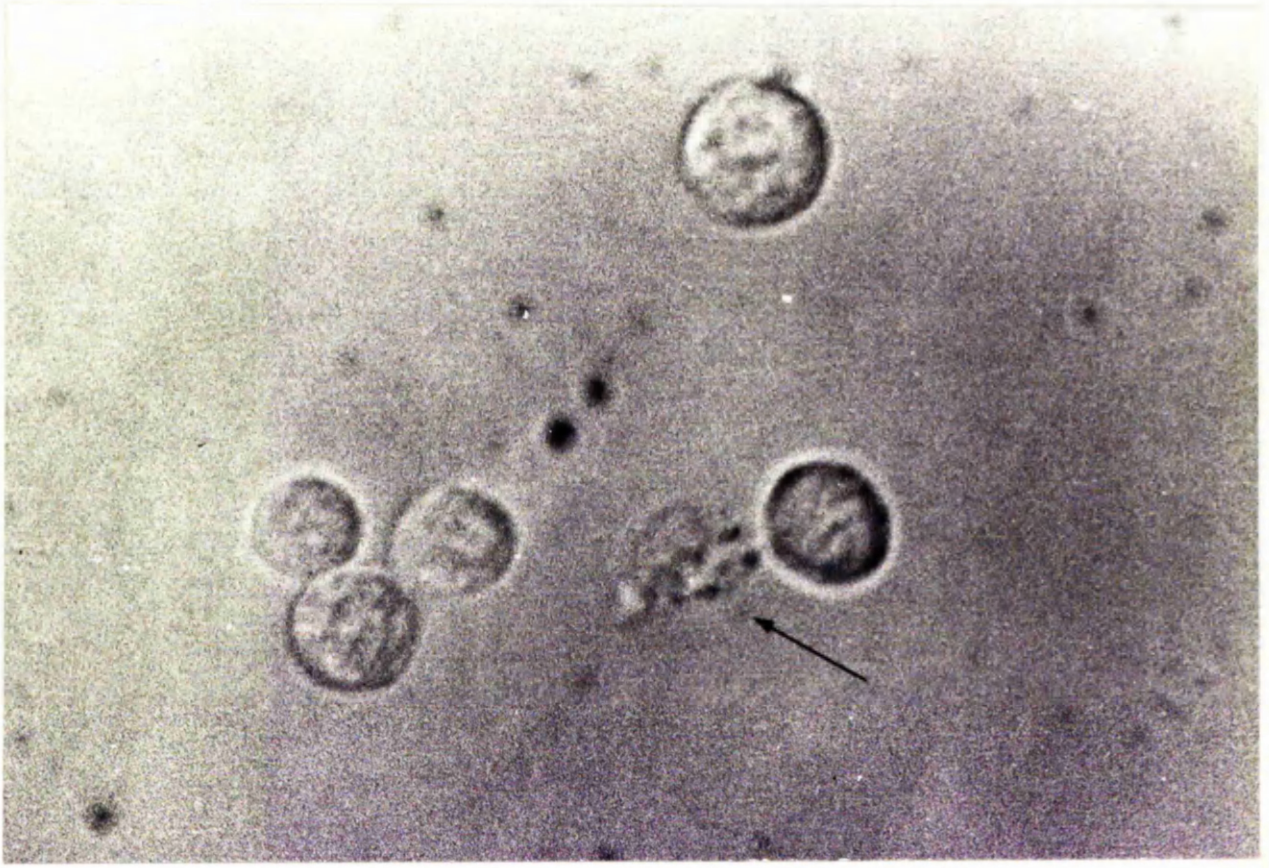


Figure 4.

A "dead" (trypan-blue staining) cell, indicated with an arrow, adhering to a healthy cell.

Brightfield illumination,

Magnification: x1800

always limited to younger cultures with little or no multi-layering. This alteration in the properties of the culture may have been caused by intercellular collagen which is produced in overgrown C-13 cultures (Elsdale and Foley 1969). The effect of EDTA in the dispersing medium is unknown, but it may help to weaken cellular adhesion.

B. The measurement of adhesion

1) Methods

(a) Agitation

Four milliliters of cell suspension in Hanks were added to stoppered, siliconized 10 ml. conical flasks. Silicon[10] treatment was used to help prevent cells from adhering to the wall of the flask. For aggregation, the flasks were removed from ice and placed in a 37°C water bath in a reciprocal shaker. Agitation was at 92 strokes per minute over a 4 cm. displacement.

(b) Sampling

For the measurement of particle population density cell suspensions were counted electronically using the Coulter counter model A[11] with a 200μ aperture. Aliquots of 0.2 ml. were removed from the cell suspension and diluted in 19.8mls. of ice cold, 0.9% sterile saline in a universal bottle before counting. As a result of this step, the applied standard coincidence correction /

correction was always less than 10% (Edwards and Campbell 1971a).

The procedures of dissociation and aggregation were monitored by hemocytometry using phase optics. This was particularly necessary to confirm that decreased total particle concentrations during aggregation were not due to cell loss by lysis.

In some experiments, the sample aliquot was collected by an automatic 0.2 ml. pipet[12] but it was found that more reproducible results were obtained using a 0.2 ml. glass micropipet. The tips of the pipets were cut off and fire polished to decrease possible degredative effects of shear on cluster size caused by too narrow an orifice.

The diluted samples were kept at $0^{\circ}C$ for not more than 30 minutes before counting. Edwards and Campbell (1971a) found that this treatment and the passage of the suspension through the Coulter aperture did not lead to disaggregation of the cell clusters.

2) The Coulter counter

In order to count total particles the minimum particle size threshold on the Coulter counter was adjusted to register only particles as large or larger than single cells. Allowance was made for any possible shrinkage of cell size during incubation (Edwards and Campbell 1971a). Thus each integral drop /

drop in the total particle number may be taken as evidence of the formation of one adhesion. At least three to five readings (of either 0.5 or 2.0 mls) were taken from each diluted sample.

Edwards and Campbell (1971a) have also found that over a wide range of particle concentration the Coulter counter responds linearly to decreases in particle number as cells aggregate. Thus it is an ideal method for measuring the concentration of particles in suspensions irrespective of particle size. The standard deviation of the particle concentration obtained electronically was 3% while that for counts by hemocytometry was about 7% (see also Miale 1962).

3) The phenomenon

In their study of the aggregation of C13 cells, Edwards and Campbell (1971a) described the rapid aggregation of these cells when they were shaken at 37°C in Hanks. The suspension quickly formed a population of single cells and clusters of various sizes and thereafter remained stable (fig.5 and 6). The final extent of the aggregation was independent of the initial particle concentration but it varied between cultures grown on different days, perhaps as a function of differences in passage number and growth. The authors found that the final extent of aggregation was strongly sensitive to the growth density /

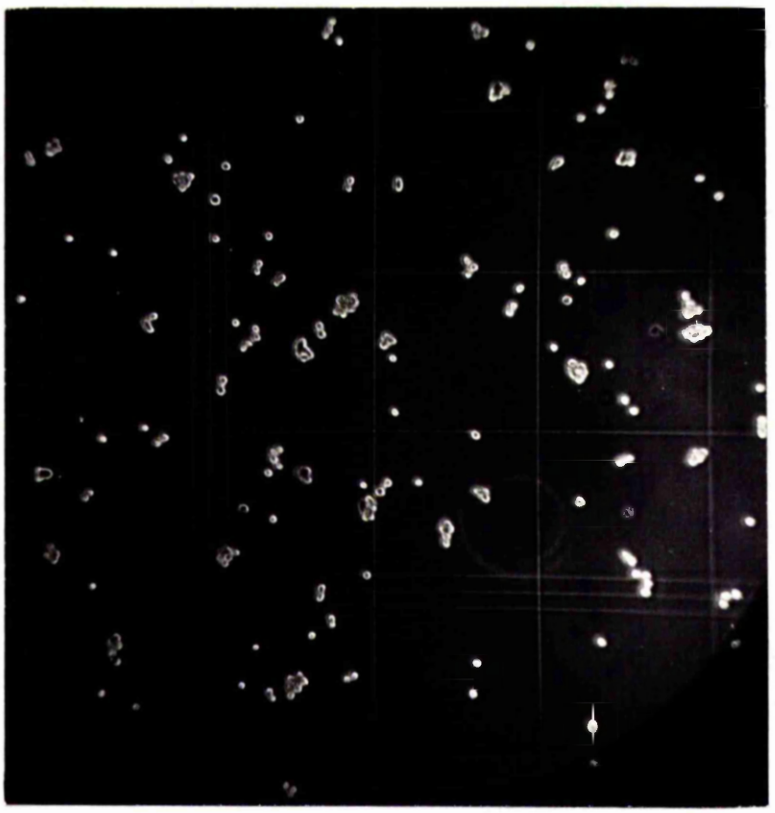


Figure 5.

An aggregated suspension after 45 minutes (ϕ = 0.379). The particles contain a distribution of cell numbers, in this case between one and ten cells/particle.

Phase optics,

Magnification: x284

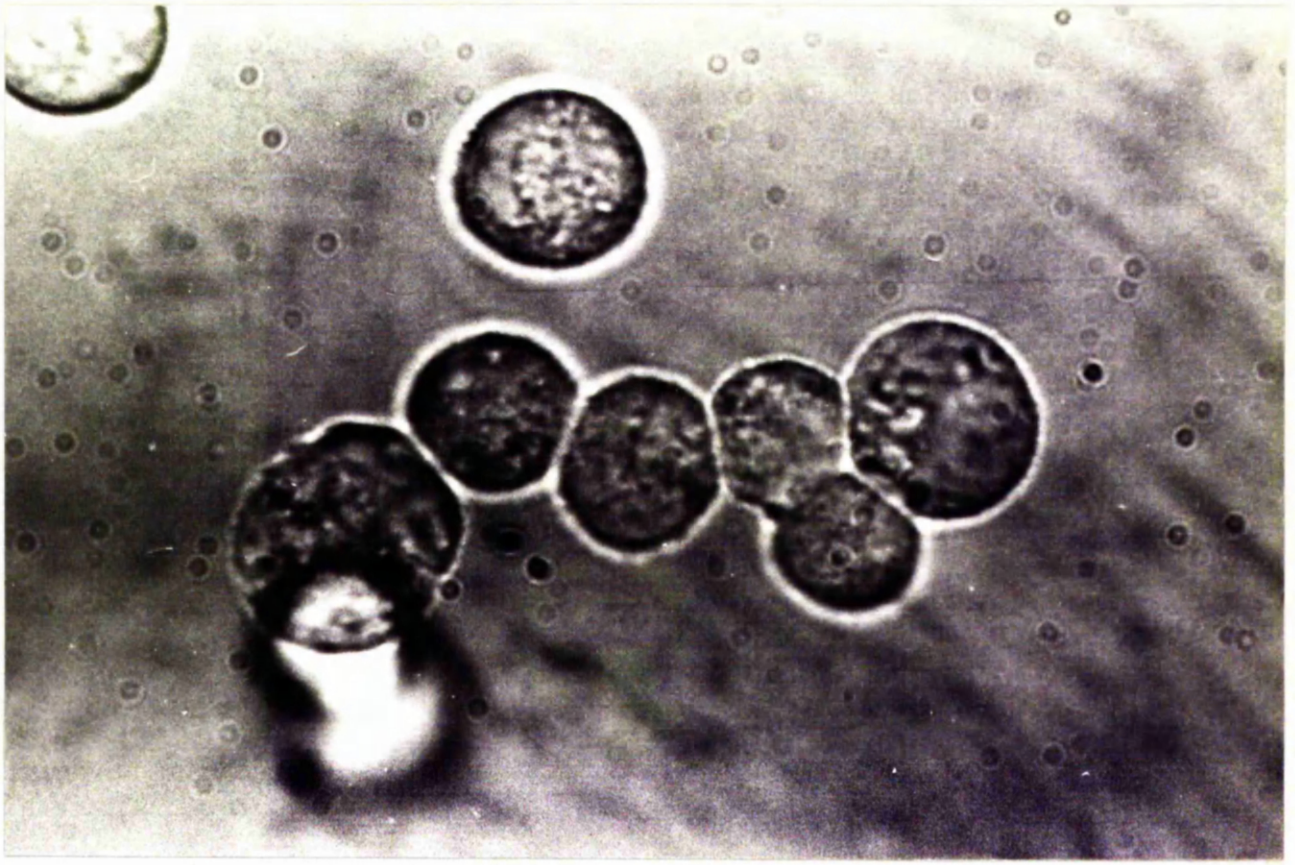


Figure 6.

Close-up of a C-13 cluster from an aggregated suspension. Notice the flattening of the cells in the area of contact. The background contains, out of focus, small particles of cellular debris and a cell cluster.

Brightfield illumination,

Magnification: x4050

density of the culture. The final extent varied from a total particle count of about one third of the initial count using densely grown cells, to almost no aggregation using very thinly grown cells.

Edwards and Campbell (1971a) found that the time necessary for the particle count to reach half-way to its final extent (half time of the aggregation) was approximately inversely proportional to the initial particle concentration as expected for a collision dependent process.

The authors also found that C-13 aggregation was temperature sensitive with little or no aggregation at 0°C. The incubation of cell suspensions at 37°C caused them to aggregate but this was found not to depend on cellular metabolism since pre-incubation at 37°C in Hanks' was ineffective in promoting aggregation at 0°C.

4) Aggregation kinetics

(a) The Model

Unlike the kinetic relationship used by Curtis (1969, eq. 2 p 49) the aggregation of C-13 cells did not fit an exponential decay function of N_t. This was because aggregation halted after a short time and N_t approached a limiting value (N_f) asymtotically as t approached ∞. The kinetics of C-13 aggregation were found to fit an exponential decay function of $N_t - N_f$.

$$N_t = N_o \left[p + (1 - p) e^{-\alpha t} \right] \qquad \text{(eq.3)}$$

where/

where N_t is the total particle concentration
at time t (o or t),
p, the proportion of N_o remaining
at N_f.
α, the pseudo-first order rate constant, and
e, the natural log base.

Derivation of equation 3:

If $\frac{dN_t}{dt} = -\alpha (N_t - N_f)$ and α is a constant

then $\int_{N_o}^{N_t} \frac{dN_t}{N_t - N_f} = -\alpha \int_{o}^{\infty} dt$

$$\ln \frac{N_o - N_f}{N_t - N_f} = -\alpha t$$

$$\frac{N_o - N_f}{N_t - N_f} = e^{-\alpha t}, \qquad N_o \cdot p = N_f$$

$$\therefore \quad N_t = N_o \left[p + (1 - p)\, e^{-\alpha t} \right] .$$

(b) Presentation of data

Two parameters are used in this study to describe the aggregation of a cell suspension. Rather than using p and the final extent of aggregation is expressed as ϕ (= 1 - p), the number of adhesions formed, and the rate constant is for a second order process, A(= α/N_o). The data are presented with ordinantes N_t/N_o in order that the ϕ values for different cell suspensions may be compared. The abscissa is time transformed /

transformed by $N_o \cdot t$ in order to compare the aggregation rates of different suspensions on the same scale.

5) Computer analysis: (with Mr.Robert Elton).

The estimation of the parameters N_o, p, α, A and ϕ in equation 3 was carried out using an iterative, least squares technique for non-linear models (Guest 1961). In this method, initial estimates of N_o, p and α are made by inspection and a series of improved approximations to the true least squares values are calculated using Taylors theorem by making linear facsimiles of the model. The KDF-9 computer facilities of the University were employed for these calculations.

The parameters and their respective standard errors were computed. In addition a best fit line transformed by Nt/N_o and $N_o \cdot t$ was produced along with the residual variance (v) and standard error of the data points about the best fit line.

In over 150 aggregations fitted to this model the standard error of the best fit line varied between 10^4 and 4×10^4 (1 - 4%) which is roughly within the 3% standard deviation of the Coulter counting technique (see tables).

C. Statistics

In order to test for significant difference in aggregation between any two suspensions, a t test may be conducted/

ducted between their parameters of ϕ or between those of A. Therefore in order to calculate t for any two curves i(1 or 2)

$$t = \frac{\pi_1 - \pi_2}{\sqrt{V\left(\frac{S_1^2}{v_1} + \frac{S_2^2}{v_2}\right)}} \qquad \text{(eq. 4)}$$

where $V = (n_1 - 3)\, v_1 + (n_2 - 3)\, v_2 \,/\, n_1 + n_2 - 6$

and π_i is the parameter N_o, α, A, p or ϕ,

S_i, the standard error,

v_i, the residual variance,

n_i, the number of data points, and

degrees of freedom (DF) = $n_1 + n_2 - 6$.

D. Divergence

Three cases arose where computation of aggregation parameters failed because there was no true minimum for the residual sum of squares.

1) No aggregation

In this case ϕ = o. N_o, and the best fit line could be calculated from the mean of the points. A and α could not be estimated. The standard error of N_o and ϕ, and the residual variance and standard error of the points about the mean, were calculated as follows:

$$SE = \sqrt{v/n} \qquad \text{(eq. 5)}$$

where /

where SE is the standard error of N_o and ϕ,

n, the number of points and

v, the residual variance, $= \left(\frac{\sum x^2}{n} - \left(\frac{\sum x}{n} \right)^2 \right)$

$x = N_t, \ldots, N_f$

2) Limited aggregation

In this case, rapid but limited aggregation ($\phi \lesssim 0.1$) occurred and not enough data was collected at early times of greatest change in the particle concentration. In these situations p was calculated from $\bar{N}_f/N_o$ where $\bar{N}_f$ is the mean of all data points ($N_{t_2} \ldots N_{t_n}$) but N_o.

The standard error of p and ϕ,

$$S_\phi \cong \sqrt{\frac{\bar{N}_f^2 \cdot V_{N_o} + N_o^2 \cdot V_{\bar{N}_f}}{N_o^4}} \qquad \text{(eq.6)}$$

where $\bar{N}_f$ is the mean of all points but N_o,

V_{N_o} the residual variance of all the points but N_o, and

$V_{N_f} = V_{N_o/n}$.

The standard error of N_o is

$$S_{N_o} = V_{N_o} \qquad \text{(eq.7)}$$

3) Linear regression

In two situations the kinetics of aggregation were anomalous and could best be fit by linear regression of N_t with t. The computation of the Bravais - Pearson coefficient of /

of regression:

$$r = \frac{n\sum N_t \cdot t - (\sum N_t)(\sum t)}{\sqrt{\left[n\sum N_t^2 - (\sum N_t)^2\right] \cdot \left[n\sum t^2 - (\sum t)^2\right]}}$$

The kinetics were fitted to the model

$$N_t = ct + N_o \qquad \text{(eq. 8)}$$

where the aggregation rate $c = \dfrac{n\sum tN_t - \sum t\sum N_t}{n\sum t^2 - (\sum t)^2}$ (eq.9)

and $N_o = \dfrac{\sum N_t - c\sum t}{n}$ (eq.10)

The table for the significance of r was used from Biometrica Tables (Pearson and Hartley 1966). The one tail values were used.

II. Proteases

A. Trypsin : introduction

Trypsin has been used to diseggregate a number of cell types as discussed previously. Moscona (1963) has shown that soya bean trypsin inhibition prevents disaggregation by trypsin. Easty and Mutolo (1960) and Edwards and Campbell (1971a) found that di-isopropylphosphoryl trypsin was inactive in dissociating tissues and aggregates. Therefore the effectiveness of trypsin appears to depend on its enzymic activity.

Trypsin dispersed cells have been shown to be capable of aggregation. In many cases, the trypsin treatment is rather extensive and protein synthesis is required for aggregation (Moscona 1968, Roth, 1968, Lillien 1969). Oppenheimer et al (1969) found that the aggregation of trypsinized teratoma cells required the synthesis of amino sugars. However, Edwards and Campbell (1971a) have found that C-13 cells were instantly adhesive following light trypsin treatment.

It therefore seemed appropriate to examine the function of trypsin in C-13 aggregation, especially in regard to the instantaneous adhesiveness and limited extent of aggregation of trypsinized cells. Special emphasis was applied to testing whether or not these phenomena were artefacts of the trypsin-EDTA /

EDTA dispersed procedure. Three types of experiments were carried out observing the effect of trypsin on aggregates and the effects of trypsin pre-treatment on cultures and cell suspensions before aggregation.

Experimental

1) Trypsin on aggregates

a) Methods

In one experiment a cell suspension was prepared using purified (10000 - 12000 BAEE units/mg) trypsin[13]-EDTA at 350 BAEE units/ml. The cells were aggregated in 8 individual flasks each containing 4 mls of Hanks' solution with .10 mg/ml. of DNAase[14] used to prevent formation of trypsin-DNA gel from broken cells (Steinberg 1963). After 100 minutes of aggregation (enough time for the system to reach a stable state) 0.1 mls. of either Hanks' or various concentrations of purified trypsin was added to each flask. After 2 minutes, of these additions, 0.05 ml. of purified soya bean trypsin inhibitor (ti) (possessing a measured inhibitory activity of 1.6 mg. ti to 1 mg trypsin)was added to each flask. The concentration of ti in each flask was 1.27 mg./ml. which was double that necessary to inhibit the largest amount of trypsin used. Purified trypsin was used because the dissociating activity of Difco trypsin was not entirely sensitive to the inhibitor/

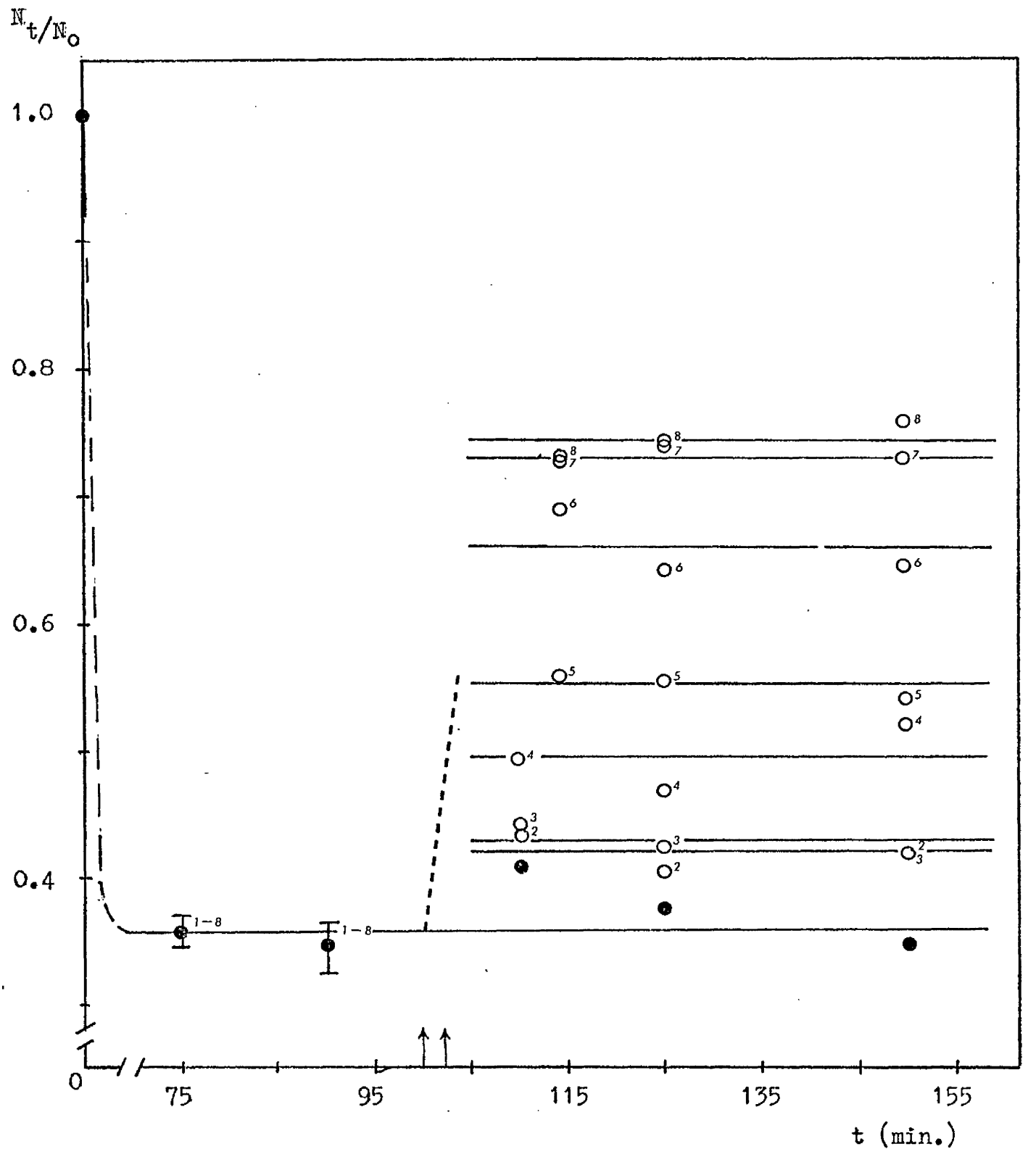


Figure 7. TRYPSIN ADDITION TO PRE-FORMED C-13AGGREGATES, exp. 1: the amount of trypsin added to each flask at room temperature,

flask:	●	2	3	4	5	6	7	8
units (BAEEunits/ml.):	0	504	980	1969	3444	4918	7372	9839;

trypsin addition at first arrow, ti addition at second arrow; lines are the average of points after ti addition and return to 37°C water bath.

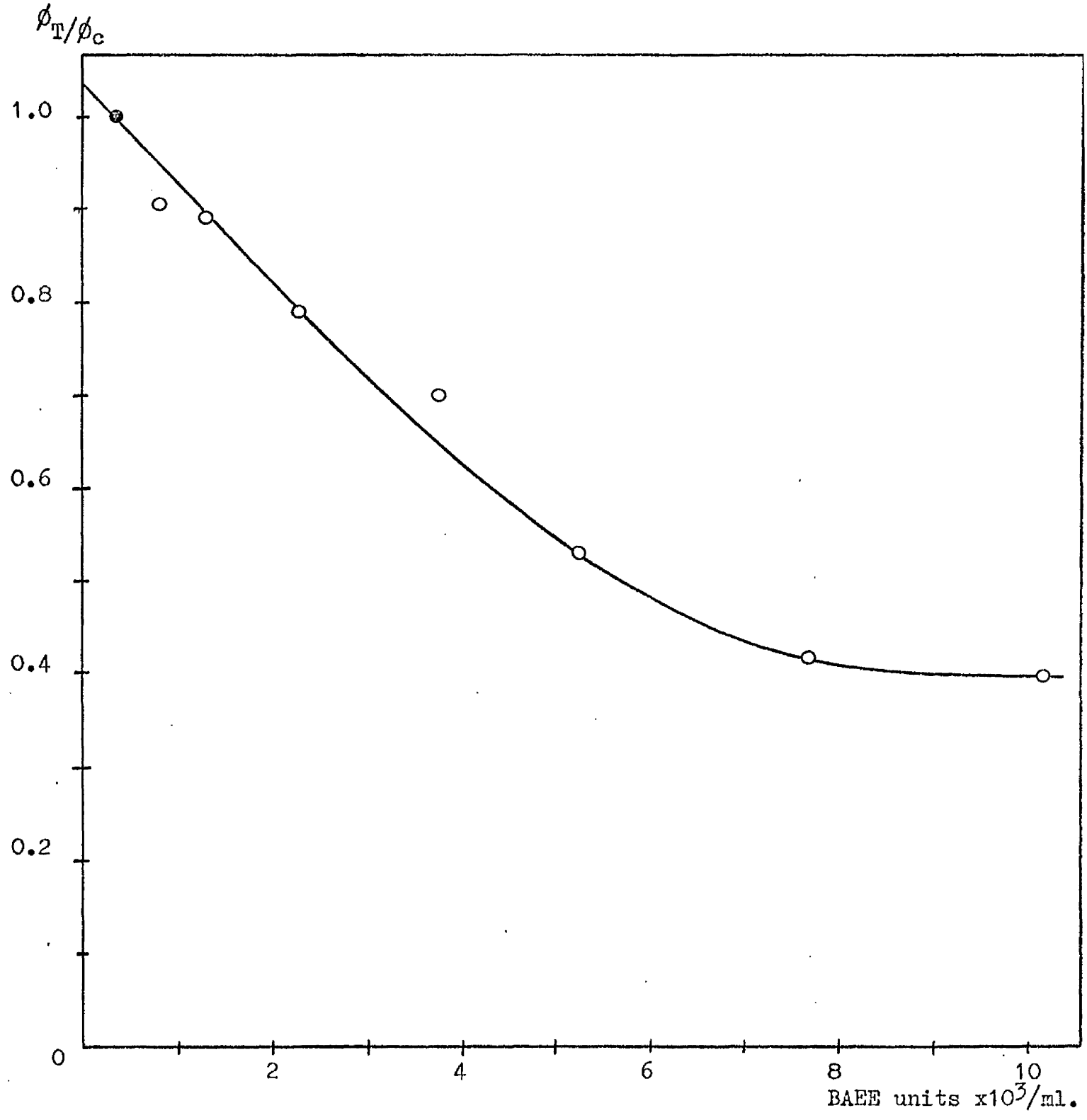


Figure 8. TRYPSIN ADDITION TO PRE-FORMED C-13 AGGREGATES, exp. 1:

Relative aggregation compared to that in the control.

The abscissa indicates the total amount of trypsin applied to the cells including that used to disperse the culture initially.

● control; o extra trypsin treated.

inhibitor probably due to the presence of other proteases.

b) Results exp.II 1*

The results are shown in Figs. 7 and 8. It can be seen that trypsin causes dissociation of aggregates as a function of the enzymic activity and time. There is no detectable re-aggregation following ti addition.

2) Trypsin pre-treatment of cell suspensions

In three separate experiments C-13 cultures were harvested with trypsin and then, as a single cell suspension, were again treated with different concentrations of trypsin before being aggregated.

a) Methods

A C-13 suspension was prepared with 0.05% Difco trypsin-EDTA and washed once in tris. The cells were then re-suspended in 5 separate 25 ml. aliquots of tris-EDTA (same concentration as before) each containing different concentrations of purified trypsin and 10 µg/ml. of DNAase. Incubation was for 2 minutes at room temperature. After this period 25 mls. of ice cold ti in tris was rapidly added and mixed and the suspensions were placed on ice. Enough ti was added in each suspension to inhibit double the amount of trypsin present. The suspensions were then centrifuged and washed once again in tris. The cell pellets were resuspended at /

* The experiments are numbered by their position in the section

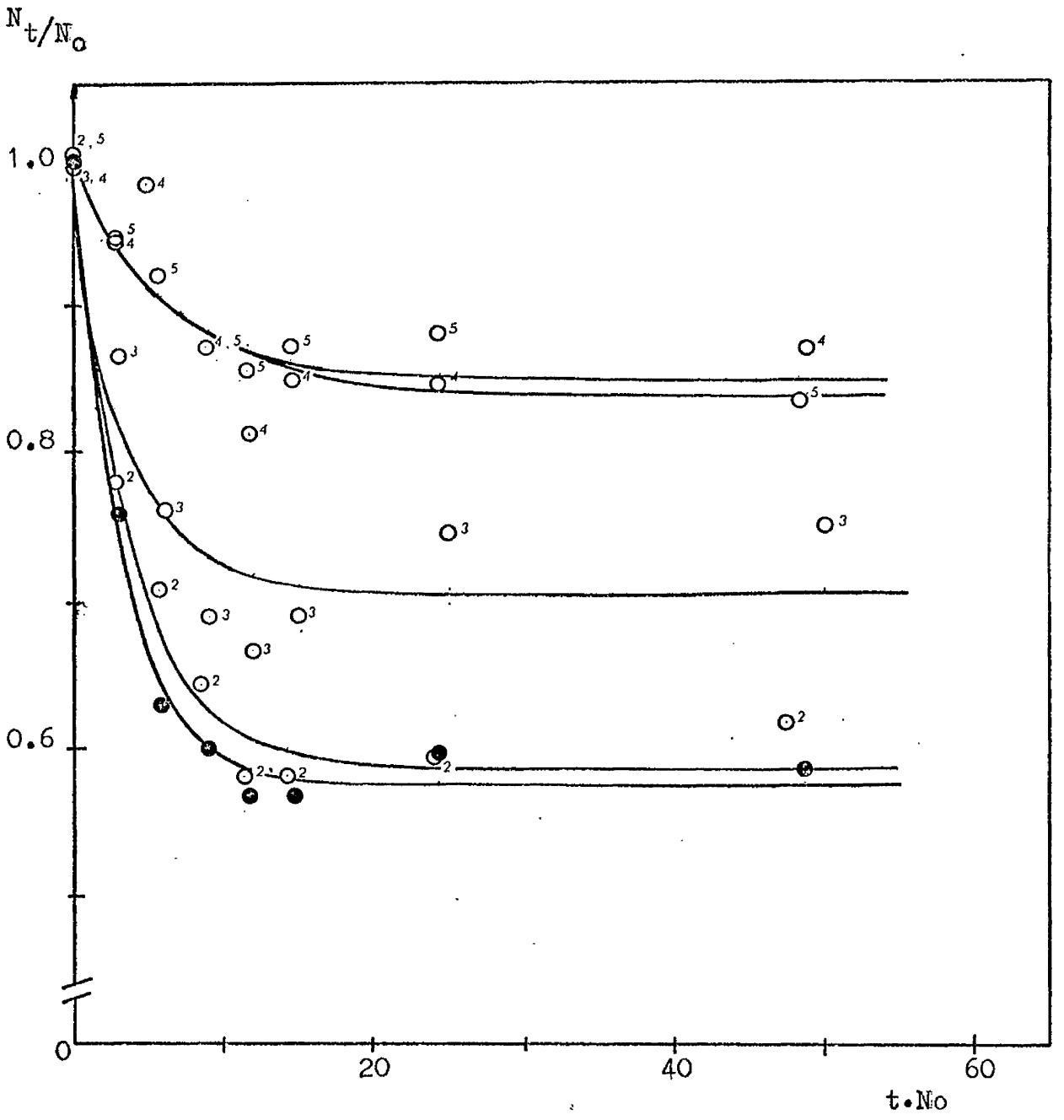


Figure 9. TRYPSIN PRE-TREATMENT OF C-13 CELLS IN SUSPENSION, exp. 3: pre-incubation of cells before aggregation at room temperature for 2',

flask:	●	2	3	4	5
BAEE units/ml.: (including the amount used to harvest the cultures);	300	690	1860	3030	4200

curves are best-fit lines.

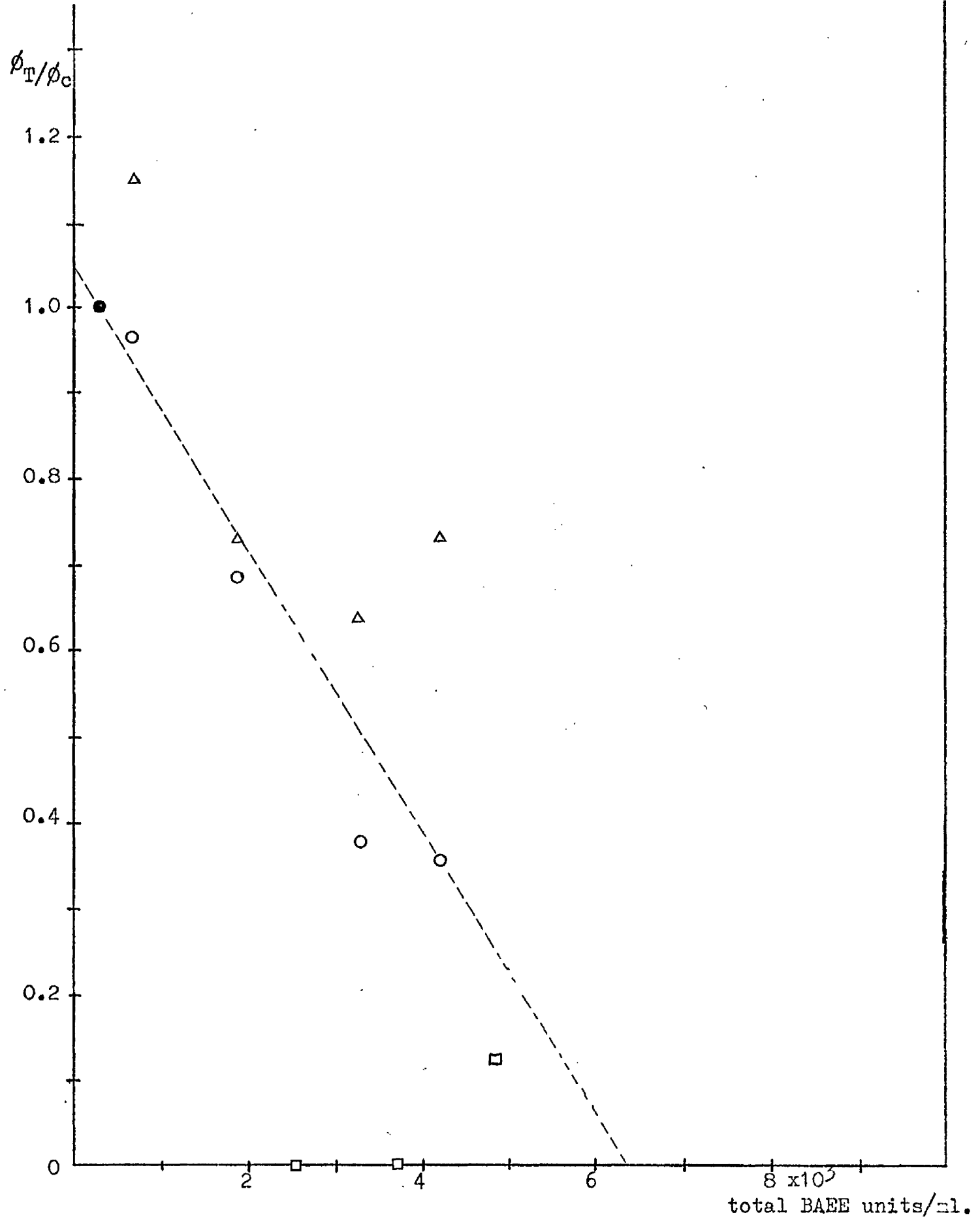


Figure 11. TRYPSIN PRE-TREATMENT OF C-13 CELLS IN SUSPENSION;
Relative aggregation compared to that in the controls,
experiment: 2 3 4
□ △ ○

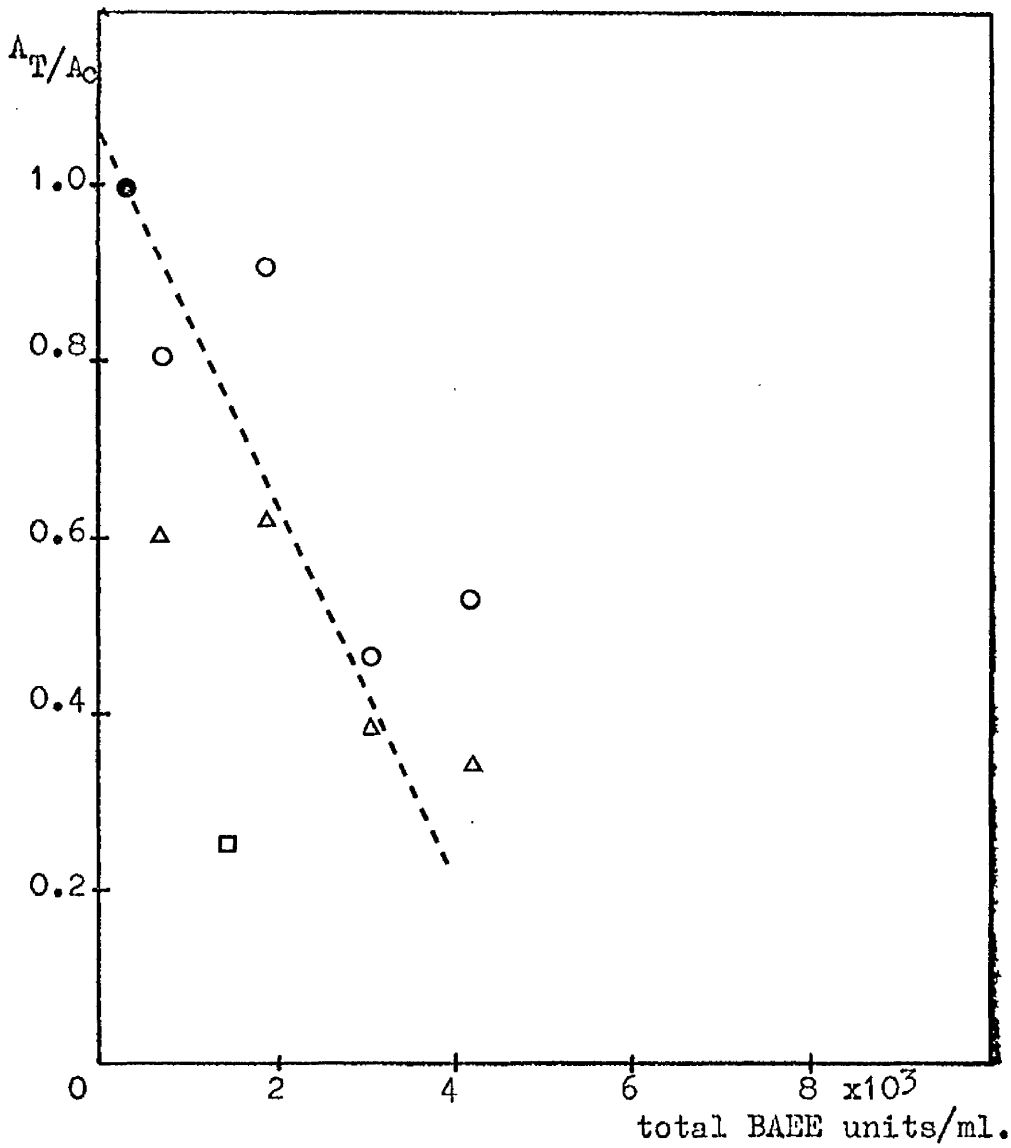


Figure 13. TRYPSIN PRE-TREATMENT OF C-13 CELLS IN SUSPENSION; Relative rate constants of aggregation compared with that in the control,

experiment: 2 □ 3 △ 4 ○

TABLE I

Pre-treatment Of C-13 Suspensions With Various Concentrations Of Trypsin

The concentration of trypsin, used to pre-treat the cells in suspension before aggregation, is given as BAEE units/ml. See text (p 114) for details. "n" refers to the number of experimental points taken. The values of the particle concentrations are reduced by 1×10^6. The Standard Errors for the parameters and SEp for the points about the Best Fit Line are included as ±.

EXPERIMENT 2: (control) 300 units/ml.;

n	No	A	α	ϕ	Residual Variance	SEp
7	0.7230 ±0.0157	0.2986 ±0.0092	0.2159 ±0.0048	0.2617 ±0.0114	0.0002345	0.0153
1428 units/ml.;						
6	0.6608 ±0.0211	0.0738 ±0.0069	0.0487 ±0.0043	0.3544 ±0.0008	0.0003879	0.0197
2556 units/ml.;*						
7	0.6683 ±0.0098	-	-	0.0000 ±0.0098	0.0006672	0.0258
3684 units/ml.;*						
7	0.6969 ±0.0058	-	-	0.0000 ±0.0058	0.0002338	0.0158
4852 units/ml.;**						
6	0.8990 ±0.0003	-	-	0.0320 ±0.0196	0.0003858	0.0196

* No detectable aggregation, see p109.

** Limited aggregation, see p110.

TABLE Continued/

TABLE I Continued:

n	No	A	α	ϕ	Residual Variance	SEp
EXPERIMENT 3: (control) 300 units/ml.;						
7	0.9910 ±0.0285	0.4917 ±0.0154	0.4873 ±0.0060	0.3355 ±0.0341	0.0008097	0.0285
690 units/ml.;						
8	1.0007 ±0.0429	0.2942 ±0.0247	0.2944 ±0.0212	0.3883 ±0.0547	0.0017979	0.0424
1860 units/ml.;						
7	0.9606 ±0.0188	0.3042 ±0.0082	0.2922 ±0.0054	0.2466 ±0.0192	0.0003466	0.0186
3030 units/ml.;						
8	0.9348 ±0.0359	0.1886 ±0.0180	0.1763 ±0.0154	0.2131 ±0.0340	0.0011690	0.0342
4200 units/ml.;						
8	0.9753 ±0.0384	0.1689 ±0.0226	0.1647 ±0.0210	0.2454 ±0.0382	0.0013209	0.0363
EXPERIMENT 4: (control) 300 units/ml.;						
8	0.9748 ±0.0168	0.3202 ±0.0101	0.3122 ±0.0083	0.4242 ±0.0217	0.0002757	0.0166
690 units/ml.;						
8	0.9504 ±0.0262	0.2566 ±0.0183	0.2438 ±0.0160	0.4111 ±0.0314	0.0006588	0.0257
1860 units/ml.;						
8	0.9995 ±0.0430	0.2913 ±0.0205	0.2912 ±0.0162	0.2913 ±0.0501	0.0018089	0.0425
3030 units/ml.;						
8	0.9755 ±0.0463	0.1480 ±0.0199	0.1443 ±0.0182	0.1573 ±0.0421	0.0018552	0.0431
4200 units/ml.;						
8	0.9698 ±0.0180	0.1693 ±0.0069	0.1642 ±0.0059	0.1485 ±0.0166	0.0002879	0.0170

at about 10^6 cells/ml. in Hanks with 0.014 mg/ml. of ti. Aggregation was at 37°C.

b) Results

The results are presented in figs.9,11 and 13 and in table 1. As in exp.1 $\emptyset$ is an inverse function of the trypsin activity during pre-treatment. The relative values of $\emptyset$ compared with that of the control ($\emptyset_x / \emptyset_c$) are shown in fig.11. Although extensive trypsin treatment caused at least a 5-fold reduction in aggregation (and in some cases aggregation was completely inhibited) the rate constant A was not reduced by more than half.

In two experiments the second trypsin treatment consisted of the same tryptic activity normally used to harvest cultures. The results show that this second treatment had little effect on the subsequent aggregation of the cells.

3) Trypsin pre-treatment of cultures

Experiment C was repeated pre-treating glass-cultured cells rather than suspensions in an attempt to detect a difference in the aggregation response of the cells which could be attributed to cell contact in culture.

a) Methods

In three separate experiments (exps. 5-7), cell cultures were treated with different amounts of trypsin before being harvested/

harvested and aggregated. The cells for each experiment were grown in five 65 cm^2 glass bottles. The growth medium was removed and the cultures washed with tris three times. Each bottle was first treated with 4 mls. of purified trypsin-EDTA (200 BAEE units/ml) for 2 minutes at room temperature. At the end of this period, 4 mls. of tris-EDTA containing 20 mg. of ti was added to the control bottle. To the remaining bottles were added tris-EDTA containing different concentrations of purified trypsin. The cultures were each incubated for a further 2 minutes before addition of 0.25 mls. of tris with 20 mg. of ti (twice the amount necessary to inhibit the largest amount of trypsin used). The cells were dispersed and washed twice by centrifugation in tris at 0°C before being resuspended in Hanks' for aggregation.

b) Results

The results are presented in figs. 10,12 and 14 and in Table 2 and are similar to those obtained by the pre-treatment of cell suspensions.

4). Discussion (exps. 1-7)

Increased trypsin treatment of aggregates, cultures or cell suspensions causes a decrease in the final extent of aggregation and, to a much lesser degree, the rate constant. The magnitude of the effect is similar in all three cases. There are at least four possible explanations of the effect of /

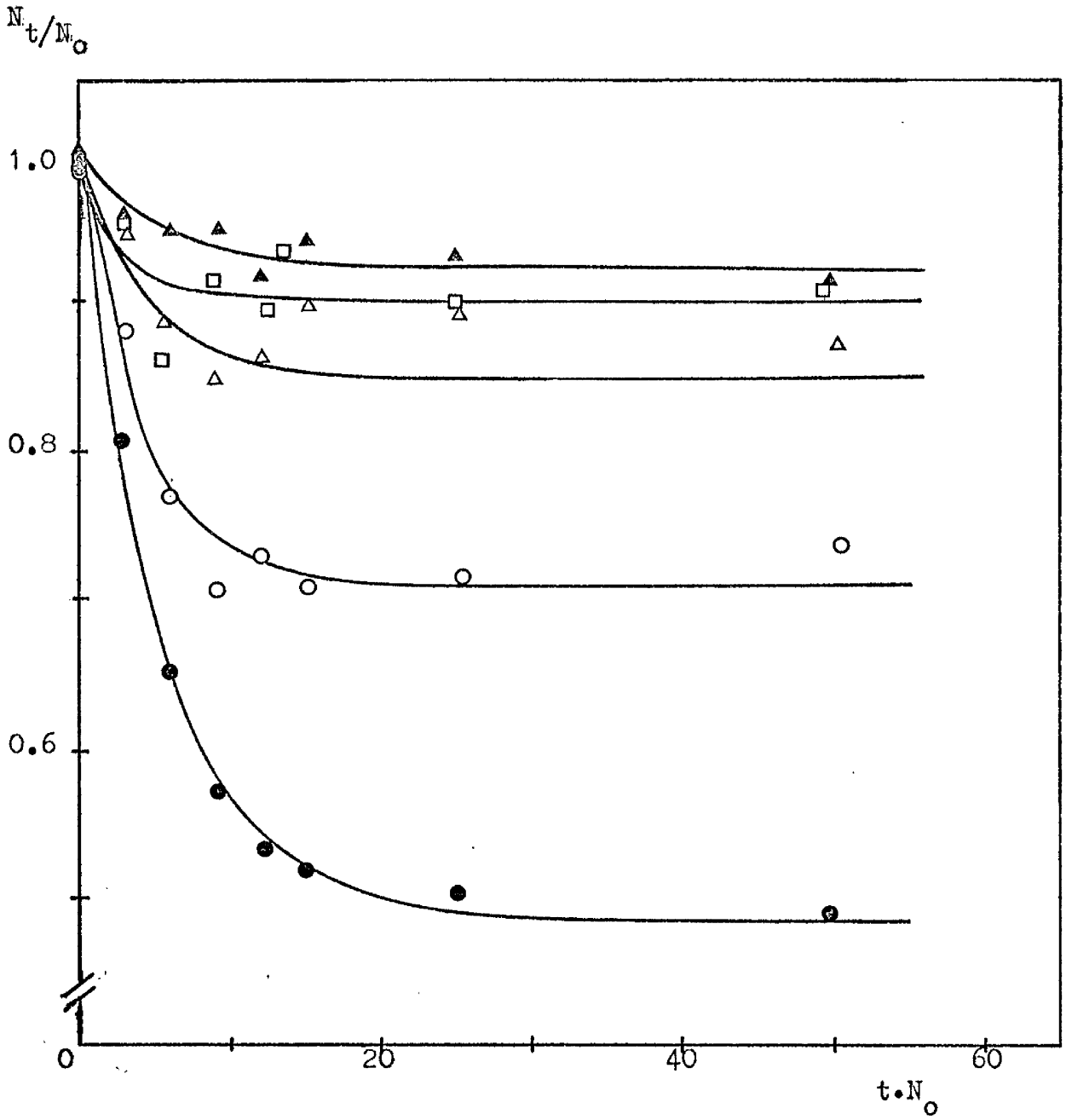


Figure 10. TRYPSIN PRE-TREATMENT OF C-13 CELL CULTURES, exp. 5: pre-incubation of cells in culture before aggregation for 2' at room temperature,

flask:	●	○	△	□	▲
total BAEE units/ml.:	175	1925	3675	5425	7175

best-fit lines.

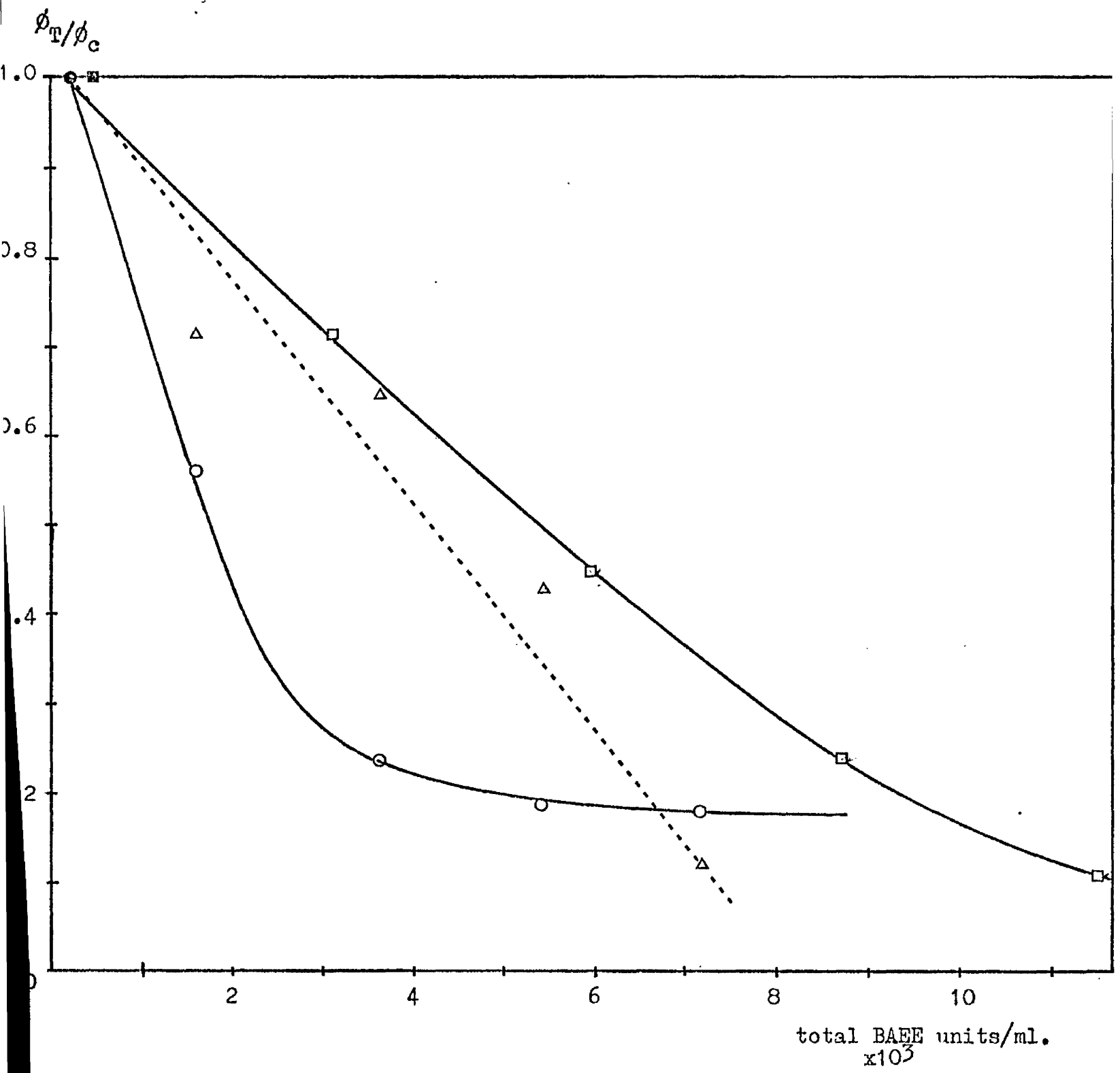


Figure 12. TRYPSIN PRE-TREATMENT OF C-13 CELL CULTURES;
Relative aggregation compared with that in the control:
experiment: 5 6 7
○ △ □

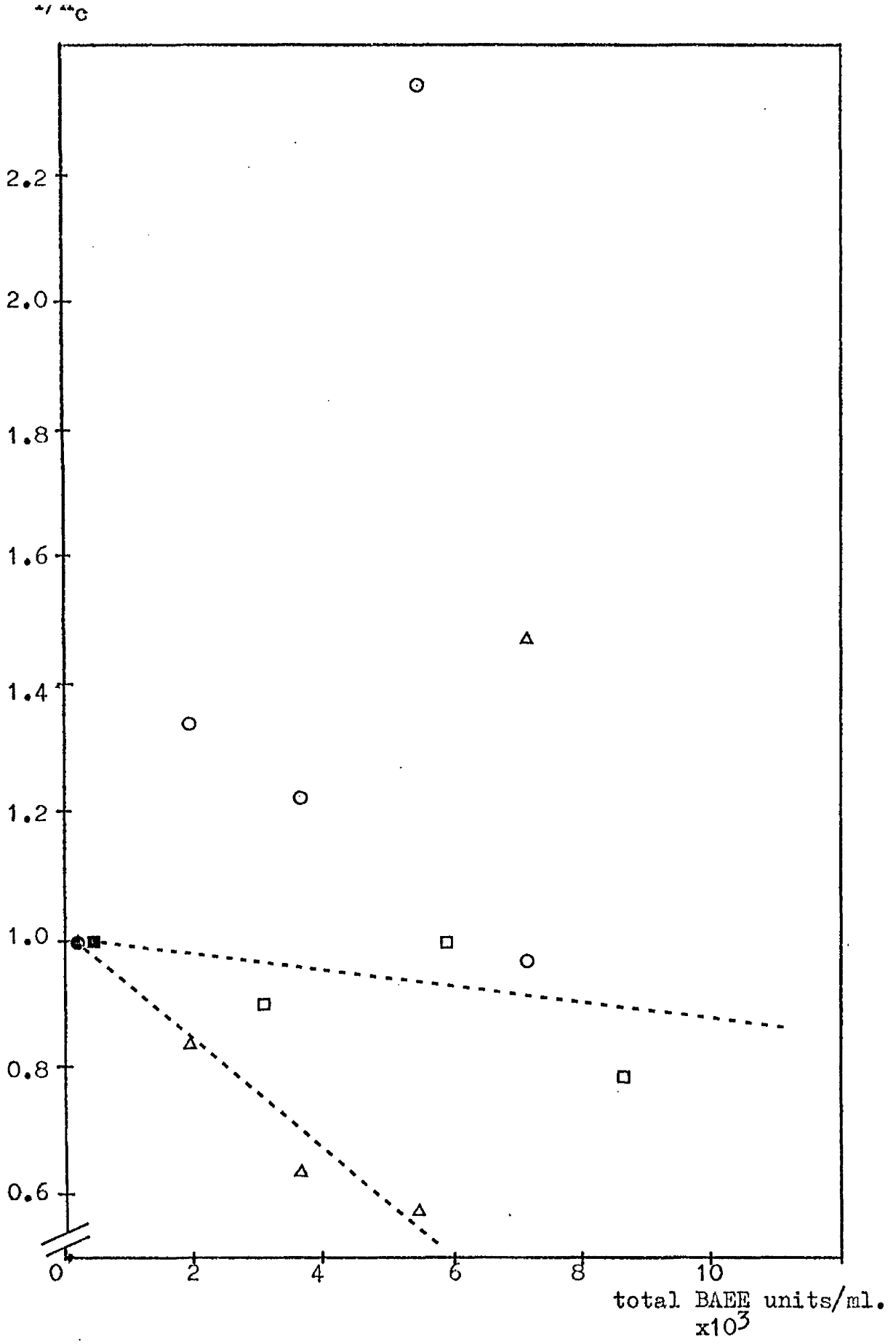


Figure 14. TRYPSIN PRE-TREATMENT OF C-13 CELL CULTURES, Relative value of the aggregation rate constants compared with that of the controls:

experiment: 5 6 7

○ △ □

TABLE 2

Pre-treatment Of C-13 Cultures With Various Concentrations Of Trypsin

The total concentration of trypsin, used to pre-treat the cells in culture before aggregation, is given as BAEE units/ml. See text (p115) for details. "n" refers to the number of experimental points taken. The values of the particle concentrations are reduced by 1×10^6. The Standard Errors for the parameters and SEp for the points about the Best Fit Line are included as ±.

EXPERIMENT 5: (control) 175 units/ml.;

n	No	A	α	ϕ	Residual Variance	SEp
8	0.9965 ±0.0158	0.1841 ±0.0171	0.1834 ±0.0168	0.5146 ±0.0198	0.0002279	0.0151
1925 units/ml.;						
8	1.0108 ±0.0263	0.2463 ±0.0132	0.2490 ±0.0117	0.2880 ±0.0303	0.0006624	0.0257
3675 units/ml.;						
8	1.0065 ±0.0181	0.2262 ±0.0061	0.2276 ±0.0046	0.1505 ±0.0184	0.0003124	0.0177
5425 units/ml.;						
8	0.9885 ±0.0297	0.4303 ±0.0131	0.4253 ±0.0022	0.0946 ±0.0292	0.0008770	0.0296
7175 units/ml.;						
8	0.9948 ±0.0121	0.1791 ±0.0029	0.1781 ±0.0020	0.0761 ±0.0111	0.0001329	0.0115

TABLE Continued/

TABLE 2 Continued:

EXPERIMENT 6: (control) 175 units/ml.;

m	No	A	α	ϕ	Residual Variance	SEp
9	1.5954 ±0.0443	0.1254 ±0.0373	0.2000 ±0.0592	0.4082 ±0.0917	0.0018139	0.0426
1925 units/ml.;						
8	1.5935 ±0.0204	0.1049 ±0.0135	0.1671 ±0.0214	0.2919 ±0.0343	0.0003714	0.0193
3675 units/ml.;						
8	1.4498 ±0.0326	0.0797 ±0.0266	0.1155 ±0.0384	0.2637 ±0.0438	0.0008645	0.0294
5425 units/ml.;						
8	1.5062 ±0.0188	0.0718 ±0.0107	0.1081 ±0.0160	0.1745 ±0.0245	0.0002811	0.0168
7175 units/ml.;						
8	1.5540 ±0.0159	0.1845 ±0.0023	0.2867 ±0.0020	0.0603 ±0.0236	0.0002480	0.0157

EXPERIMENT 7: (control) 250 units/ml.;

8	0.9310 ±0.0181	0.2656 ±0.0115	0.2473 ±0.0095	0.3678 ±0.0205	0.0003134	0.0177
3070 units/ml.;						
8	1.0280 ±0.0355	0.2418 ±0.0165	0.2486 ±0.0147	0.2626 ±0.0407	0.0012086	0.0348
5900 units/ml.;						
8	0.9928 ±0.0256	0.2648 ±0.0091	0.2629 ±0.0060	0.1633 ±0.0262	0.0006344	0.0252
8700 units/ml.;						
8	0.9528 ±0.0227	0.2079 ±0.0068	0.1981 ±0.0045	0.1077 ±0.0207	0.0004765	0.0218
11500 units/ml.;*						
8	0.9500 ±0.0101	-	-	0.0397 ±0.0374	0.0014000	0.0374

* Limited aggregation, see p110.

of trypsin and dispersal on C-13 aggregation (see diagram p.119)

1) Trypsin, or trypsin-ti complex, may promote cell aggregation by acting as an intercellular cement. The enzyme may stick to cells, in miniscule quantities difficult to detect, during the dispersal procedure.

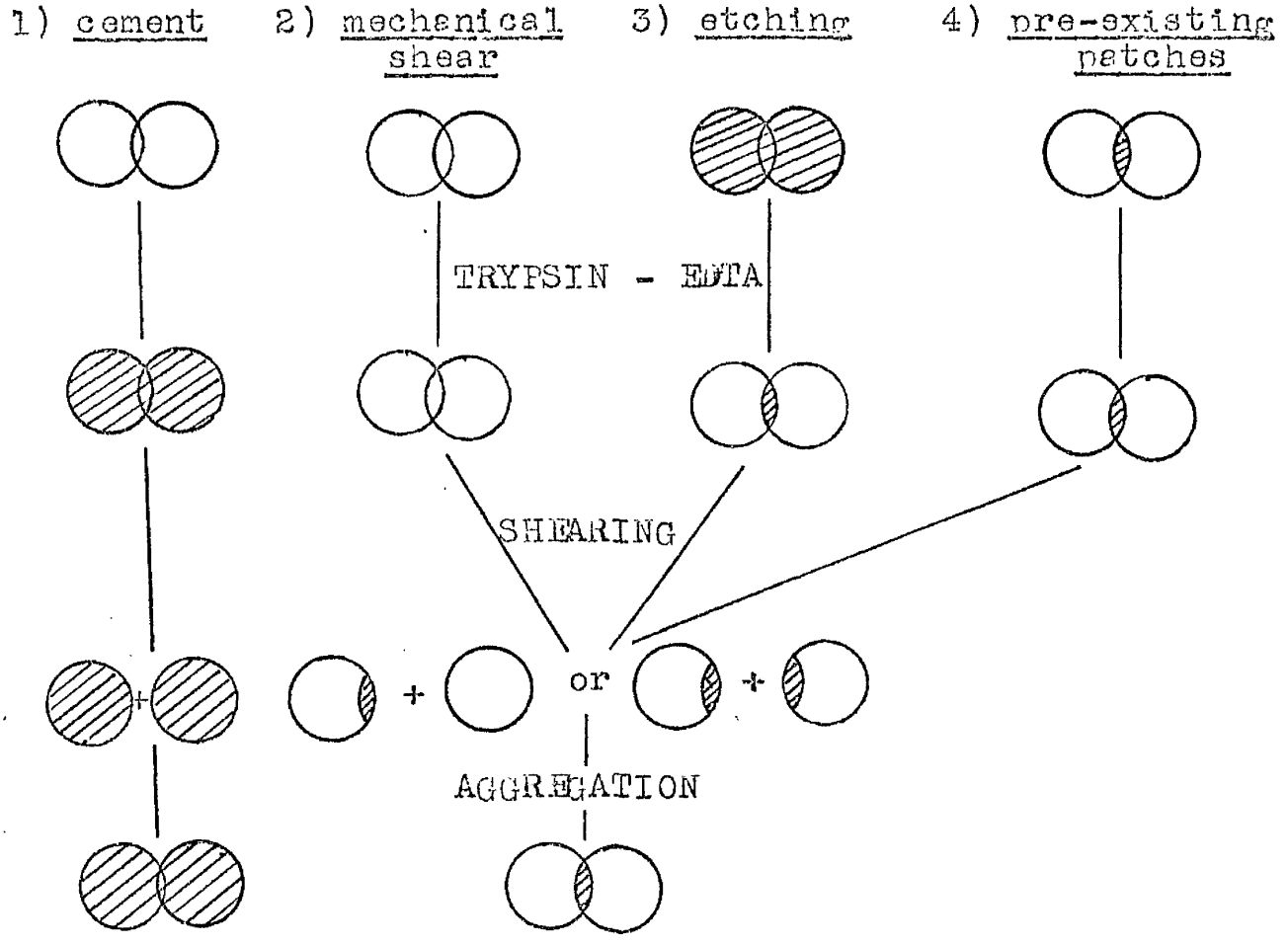
2) Although the trypsin-EDTA treatment may weaken cell contacts mechanical disruption of the junctions may modify the cell surface at these points. One way in which this might occur in heart tissue has been described by Muir (1967). Following the disruption of intercellular contacts, holes in the surface of some cells could be found and pieces of plasma membrane from one cell remained attached to the surface of another cell especially at sites of small gap junction. Therefore the strength of this junction may be greater than that of the membrane. Edwards and Campbell (1971a) have suggested that the cell surface of cells in culture could be non-adhesive but that surface modified by mechanical destruction of contacts might become adhesive. In this case, the adhesions formed during aggregation in suspension could be considered reassemblies of the former sites of contact. However, the mechanism of adhesion would probably be different and might possibly be distinguished from the mechanism of adhesion in culture.

3)/

3) Trypsin may digest the molecular adhesive components from the cell surface but the enzyme may be inhibited from entering spaces of close cellular contact. This possibility is realistic considering the short time (2 minutes) of exposure and the dimensions of the contacts themselves. The enzyme would then be free to etch away exposed cell surface leaving these areas non-adhesive while protected areas of contact would escape attack. Thus parts of cells in contact and all the surface of isolated cells would be attacked and the result would be a distribution of adhesiveness between cell in the population and geometrically over the cell surface of adhesive cells.

4) The adhesive patches may pre-exist on the cell and be responsible for adhesion in culture and in suspension. Trypsin may then be expected to reduce adhesiveness if the enzyme is used in increasing concentration.

1) cement/



The first three possible explanations are not supported by the evidence.

a) Mechanical injury and cementing by trypsin or trypsin-ti may be ruled out first, because aggregates formed from trypsin dispersed cells are themselves dispersed by trypsin. Therefore, the mechanism of adhesion in suspension cannot be distinguished from that causing adhesion in culture by this particular criterion of trypsin sensitivity. This conclusion /

conclusion is supported by the (probably) linear relationship between the amount of trypsin applied and the degree of subsequent aggregation (figs. 8,11 and 12). Unless there is a radicelly different slope of the curve near the point of zero trypsin concentration it may be concluded that the cells aggregate by the same trypsin-sensitive mechanism for all trypsin concentrations used and for zero trypsin. The cementing model is further ruled out because cultures or aggregates in the presence of trypsin-ti complex neither disperse nor re-aggregate (exp.1, fig.7).

b) The etching model is unlikely because a second treatment of harvested cells with an equivalent concentration of trypsin causes very little decrease in the value of $\emptyset$ (fig.11 and table 1). In addition, the $\emptyset x/\emptyset_{cs}$ relationships (figs.8, 11 and 12) show that the slope of aggregation extent versus tryptic activity is similar (as far as can be seen here) whether the cells are treated as aggregates or pre-treated as suspensions or cultures. From these observations, it appears that trypsin may gain access to all areas on the cell surface even if the cells are in adhesive contact. Otherwise the slopes in figs. 8 and 12 would have been much flatter than that in fig.11.

c) Therefore, if the sensitivity curves (figs.8 and 11-14) can be extrapolated, the aggregation of C-13 cells is not /

not an artefact of the trypsin dispersal treatment. These observations cannot be used as proof for the existence of a geometrical distribution of adhesiveness in the cell population and/on the cell surface. However, it may be concluded from the results that if there are such adhesive patches, they probably pre-exist in the culture. The degree to which the adhesiveness measured here functions in normal cell behaviour in the culture and in the animal is unknown. Conceivably, other mechanisms of adhesion may operate and be of primary importance in normal cellular interactions.

The limited extent of C-13 aggregation cannot be attributed to any obvious or distinct fraction of cell population such as dead cells. First, there were few dead, trypan blue staining cells in the suspensions used in the aggregations (approximately 1-20%). Secondly, dead cells were usually included in the formation of aggregates. The origin of the non-adhesive cells in the population and of adhesive patches will be discussed later. The effect of EDTA on aggregation is also included later in this section.

B. Collagenase

1) Introduction

Collagenase has often been used in attempts to dissociate tissues into single cells. The enzyme has been applied with some success to disperse liver cells (Laws and Strickland 1961 mammary/

mammary gland tissue (Lesfarges 1957), and lung tissue (Hinz and Syverton 1959, Grover 1962). Houba (1967) found that crude collagenase was slightly effective in dispersing clots of spleen cells and erythrocytes. However, Rinaldini (1958) found that the enzyme was not readily effective in dispersing early embryonic tissue and suggested that much of the activity of crude collagenase preparations was due to contaminating proteases.

Elsdale and Foley (1969) found that human lung fibroblast and C-13 monolayer cultures do not normally produce collagen. However, as the cells become overgrown and begin to multilayer, they synthesize collagen extensively and use the extracellular collagen as new substrate which they colonize.

2) Methods

The possible dependence of intercellular adhesion on collagenase was tested by adding purified collagenase[16] to suspensions of C-13 aggregates. The activity of the enzyme preparation was tested by Professor Curtis, using the method of Grassman and Nordwig (1960) and was found to release 0.733μM peptide/min./mg. of enzyme from the substrate.

In three separate experiments (exps. 8-10) C-13 suspensions in Hanks' were divided into 4 ml. aliquots in individual 10 ml. flasks and aggregated for 80-135 minutes which was sufficient time for the system to become stable.

Each /

Each flask contained 10 µg/ml. DNAase. At that time, one of the following solutions in 0.1 ml. of Hanks was added to each flask: Hanks, collagenase or purified trypsin[17]. The effect of the additions was followed either by hemocytometry, counting single cells/ml.,or by use of the Coulter counter, measuring total particles.

3) Results

The results are shown in figs.15 and 16. There is some slight dissaggregation of the cell clusters with the highest concentrations of collagenase used, compared to the Hanks and trypsin controls. This small effect could be attributed to residual contamination of the enzyme with other proteases since any cell surface collagen would be adequately digested by the lower concentrations of collagenase used. Moscona et al (1965) has suggested that collagenase may be effective in tissue dissociation by freeing cells trapped in a collagen matrix.

C. Pronase

1) Introduction

Edwards and Campbell (1971a) found that C-13 aggregates were dissociated by trypsin and chymotrypsin as a function of proteolytic activity. Takeuchi and Yabuno (1970) have used trypsin and pronase to dissociate the slime mold grex, but they /

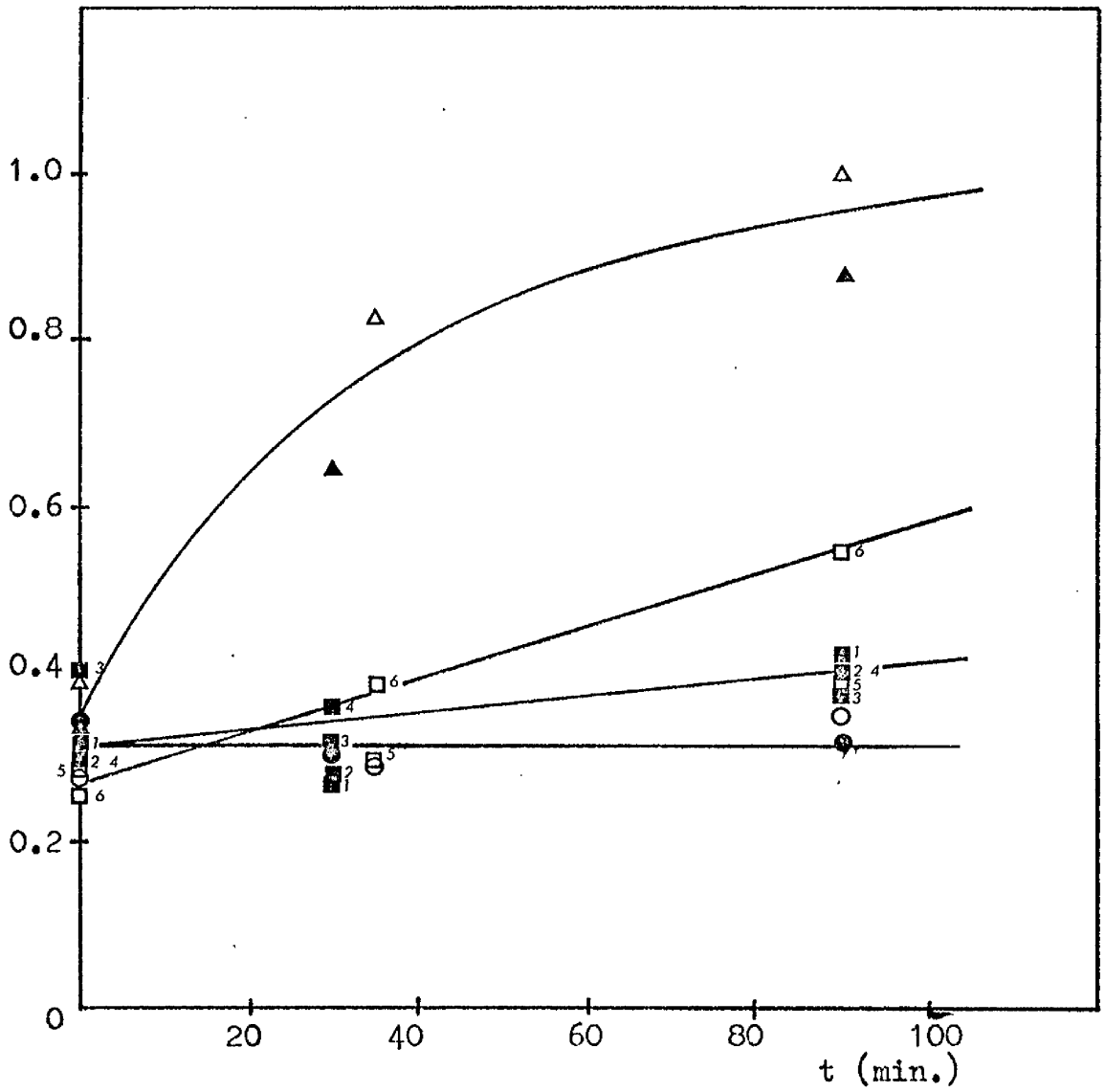


Figure 15. COLLAGENASE ADDITION TO PRE-FORMED C-13 AGGREGATES, exp.8,

abscissa - time after addition of the enzyme,
ordinate - the residual single cells in a stable, aggregated suspension composed of single cells and clusters of various sizes, counting by hemocytometry, 37°C water bath,

flask:	control		trypsin		collagenase					
	●	○	▲	△	■				□	
					1	2	3	4	5	6
concentration: (μg/ml.)	Hanks'		9.2	9.0	1.2	12.2	61.0	122.0	300.0	1000.0

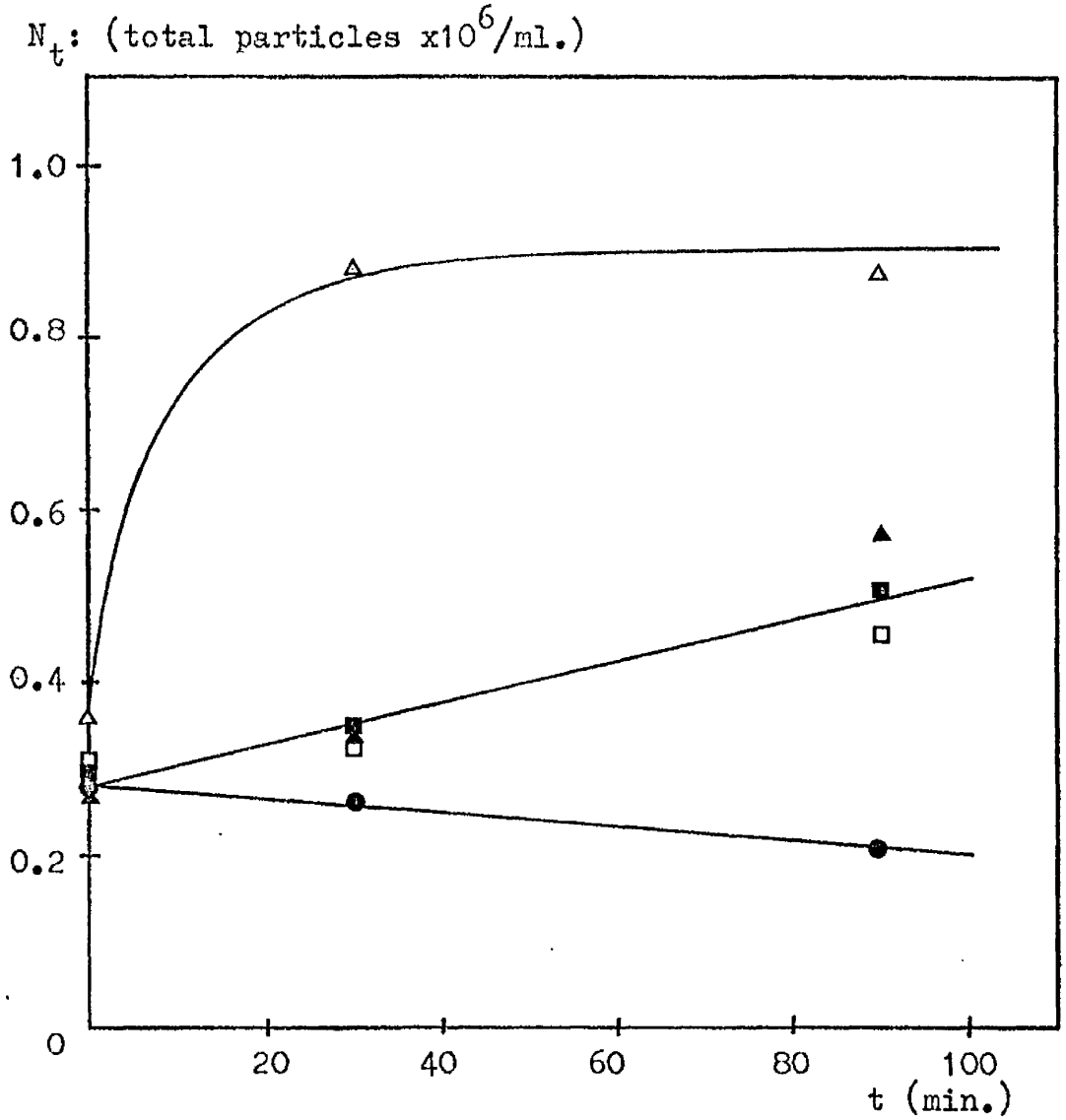


Figure 16. COLLAGENASE ADDITION TO PRE-FORMED C-13 AGGREGATES, exp.

abscissa - as fig. 15,
ordinate - N_t counted electronically,

flask:	control	trypsin		collagenase	
	●	▲	△	■	□
concentration: (μg/ml.)	Hanks'	0.18,	9.0	500	250

they found pronase to be more effective. Banks et al (1970) found that pronase was the most effective of the proteases used in dissociating embryonic chick ganglia. Chick embryonic cells have been dispersed with pronase (Wilson and Lau 1963) and the enzyme has been used to disperse mammalian tissues (Gwatkin and Thomson 1964). Pronase has been used to reduce the size of the glycopeptide fragments cleaved by trypsin from the cell surface (Meezan et al 1969).

2) Methods

In one experiment, (exp.11) C-13 aggregates were treated with pronase[18]. Cell suspensions (4 mls./flask) were aggregated for 85 minutes in Hanks' with 10µg/ml. DNAase. After this time, the suspensions ceased aggregation and the following solutions in 0.1 ml. of Hanks' were added: Hanks', purified trypsin[17] or pronase. There was no evidence of cell damage caused by pronase. The enzymic activity was not assayed.

3) Results

Pronase appears to be at least as effective as purified trypsin in dispersing the aggregates (fig.17)

D. Phospholipase C

1) Introduction

This specific enzyme hydrolyses lecithin or sphingomyelin to form a diglyceride and phosphoryl-choline. Other related lipids /

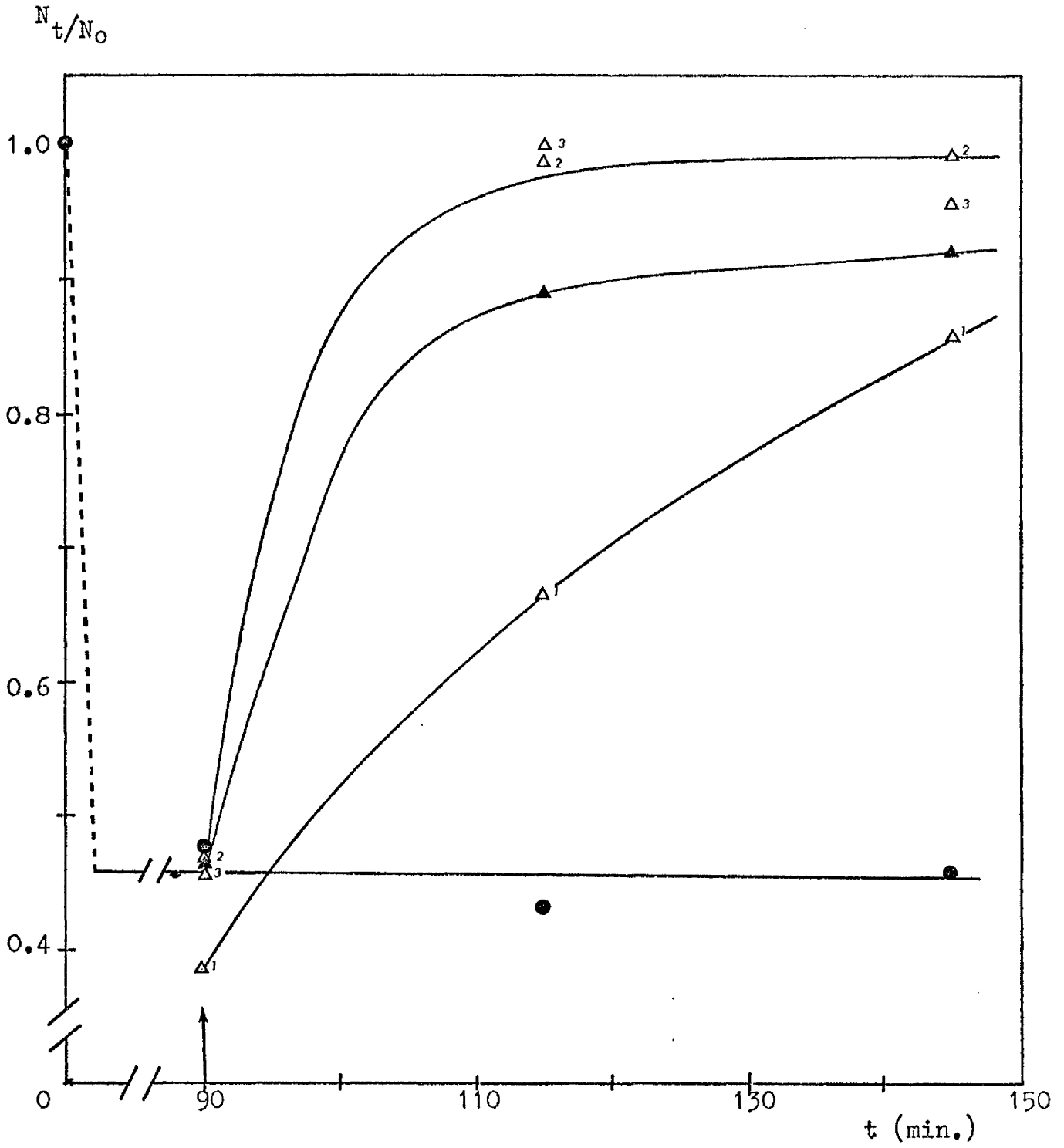


Figure 17. PRONASE TREATMENT OF PRE-FORMED C-13 AGGREGATES, exp. 11: arrow indicates time of addition of enzyme,

flask:	control	trypsin	pronase		
	●	▲	△		
			1	2	3
concentration: (μg/ml.)	Hanks'	10.0	5.4	10.8	21.5

lipids are not attacked (Hanahan and Vercamer 1954). Phospholipase C (phl C) releases phosphorylcholine from the membrane of intact erythrocytes (de Gier et al 1961). Lenard and Singer (1967) found that the enzyme released about 70% of the phosphate from erythrocyte ghosts without disturbing the integrity or structure of the membrane as detected by physical methods (see previously).

The zwitterionic head of lecithin has no net effect on the ζ potential of particles of lecithin suspended in water, but the addition of divalent cations gives the particle a positive ζ potential (Bangham and Dawson 1962). The removal of phosphorylcholine from the cell surface may, if anything, enhance the cells net negative surface charge. In addition, the number of divalent cation binding sites (e.g. phosphate) will be reduced (Shah and Schulman 1967).

2) Methods

a) Enzyme Assay

The activity of the enzyme was measured by a titrimetric method similar to that of Hanahan and Vercamer (1954), the only differences being that, whereas their assay was conducted in an ether-alcohol mixture, the system used here was entirely aqueous (Macfarlane and Knight 1941); and the titration and recording were carried out autometically[19].

Egg/

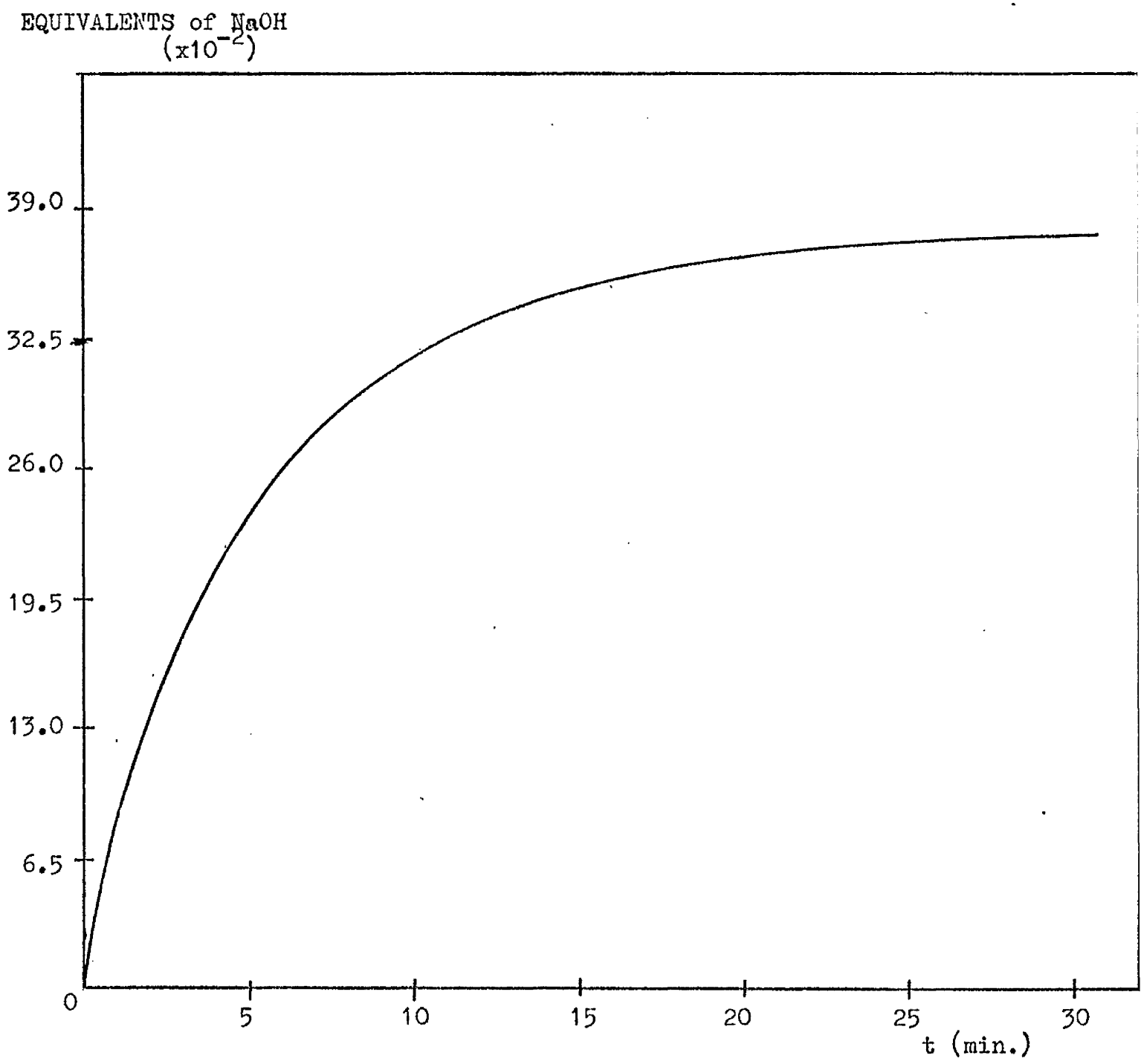
Egg yolk lecithin[20] was obtained dissolved in hexane. The solution was rotary evaporated and the lecithin re-dissolved in tetrahydrofuran (THF)[21] at about 20 mg./ml. The reaction mixture consisted of 0.75 mls. of 0.01 M $CaCl_2$[22] (final concentration 2.5 mM), lecithin in THF from 0.05 to 0.15 mls. (final concentration 0.033 to 0.1 mg./ml.), and distilled H_2O to make a final concentration of 3.0 mls. at pH 7.3.

The mixture was thermostated to 37°C (at room temperature or 26°C the mixture was turbid and the rate of reaction barely detectable). The reaction vessel was equilibrated with pure nitrogen gas[23].

The reaction was initiated by the addition of 0.05 ml. of phospholipase C[24], 2 mg./ml. in 2.5 mM $CaCl_2$ solution and titrated with $N/_{100}$ NaOH[25] also dissolved in 2.5 mM $CaCl_2$. The burette was calibrated by adding known amounts of HCl[26].

The estimated initial rate of reaction varied with the concentration of substrate. Virtually all the lecithin was hydrolyzed at rates comparable or faster than those reported by Macfarlane and Knight (1941) and Hanahan and Vercamer (1954). Disc electrophoresis of phl C in acrylamide gels showed several proteins of molecular weight greater than 3×10^5 in the phl C solution (See Appendix II).

b) /



TITRIMETRIC ASSAY OF PHOSPHOLIPASE C:

addition of 0.1ml. lecithin.

b) Effect on Aggregates

Cell suspensions were prepared as usual and aggregated in Hanks (4 mls./flask) with 10µg/ml. DNAase for 90 or 135 minutes. At this time phl C, Hanks or purified trypsin[17] was added in different amounts each less than 0.1 ml. of Hanks'. Cells appear unharmed by the enzyme at 1×10^{-3} mg./ml. but concentrations of 1×10^{-2} mg./ml. or greater caused extensive damage after 30 minutes of incubation.

3) Results

The results of two experiments (exps.12,13) are seen in figs.18 & 19. There seems little, if any, effect of phl C on C-13 aggregates.

E. EDTA.

1) Methods

In one experiment a C-13 suspension was prepared as usual but the final re-suspension in Hanks was omitted. Instead, the cells were re-suspended in a small volume of tris and aliquots of this were re-suspended at about 10^6 cells/ml. in 4 mls. of either Hanks', tris or different concentrations of EDTA. The pH of the suspensions was tested before and after aggregation and remained between 7.1 and 7.2 in each flask.

2) Results

The aggregation and data for the experiment can be seen in fig.20 and table 3. The presence of as much as a 0.01 M concentration /

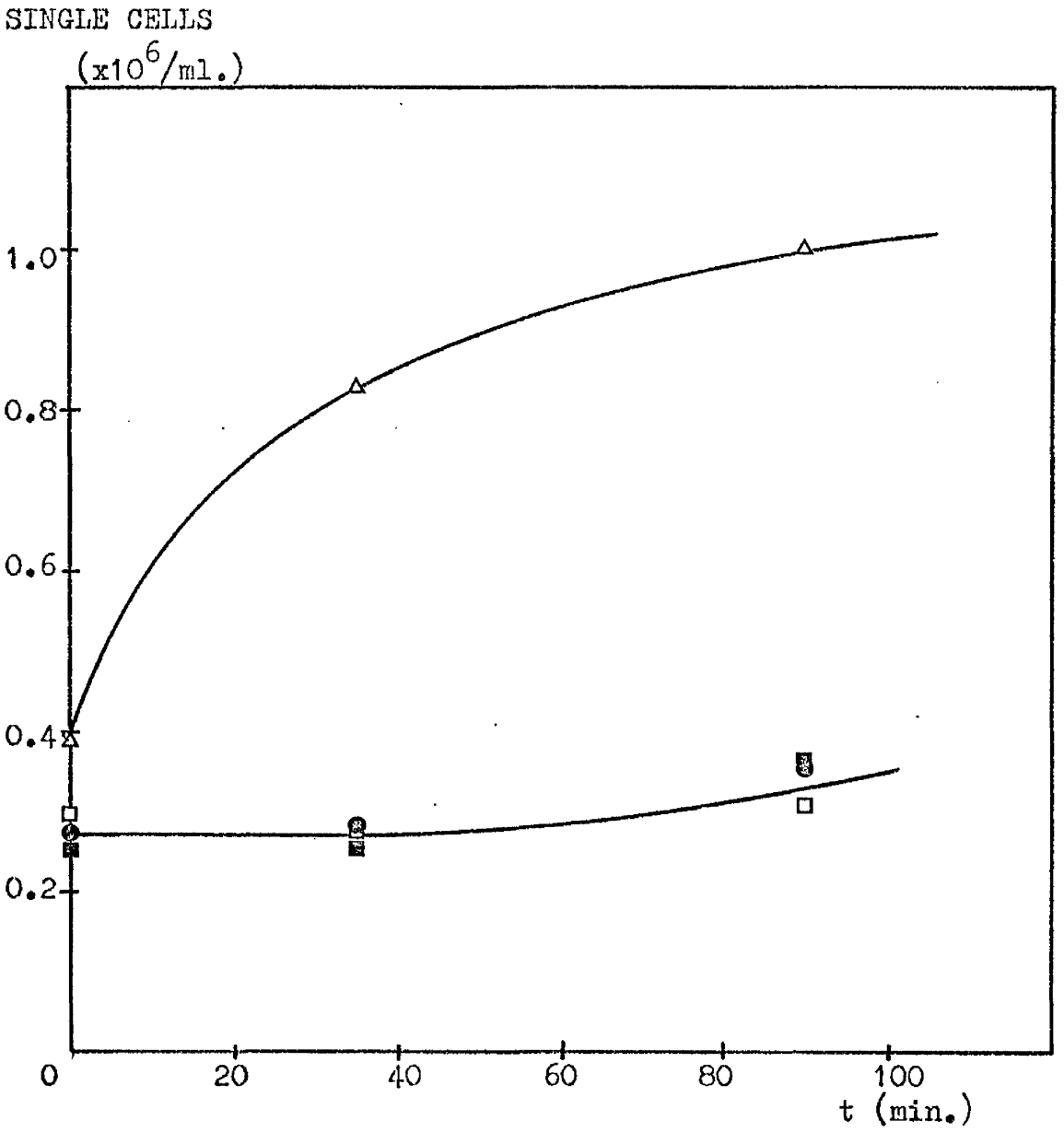


Figure 18. PHOSPHOLIPASE C TREATMENT OF PRE-FORMED C-13 AGGREGATES, exp. 12:

abscissa - time after addition of enzyme,
ordinate - concentration of residual single cells in an aggregated suspension composed of single cells and clusters of various sizes,

counting by hemocytometry,

flask:	control	trypsin	phospholipase C	
	●	△	□	■
concentration: (μg/ml.)	Hanks'	9.0	0.5	1.0

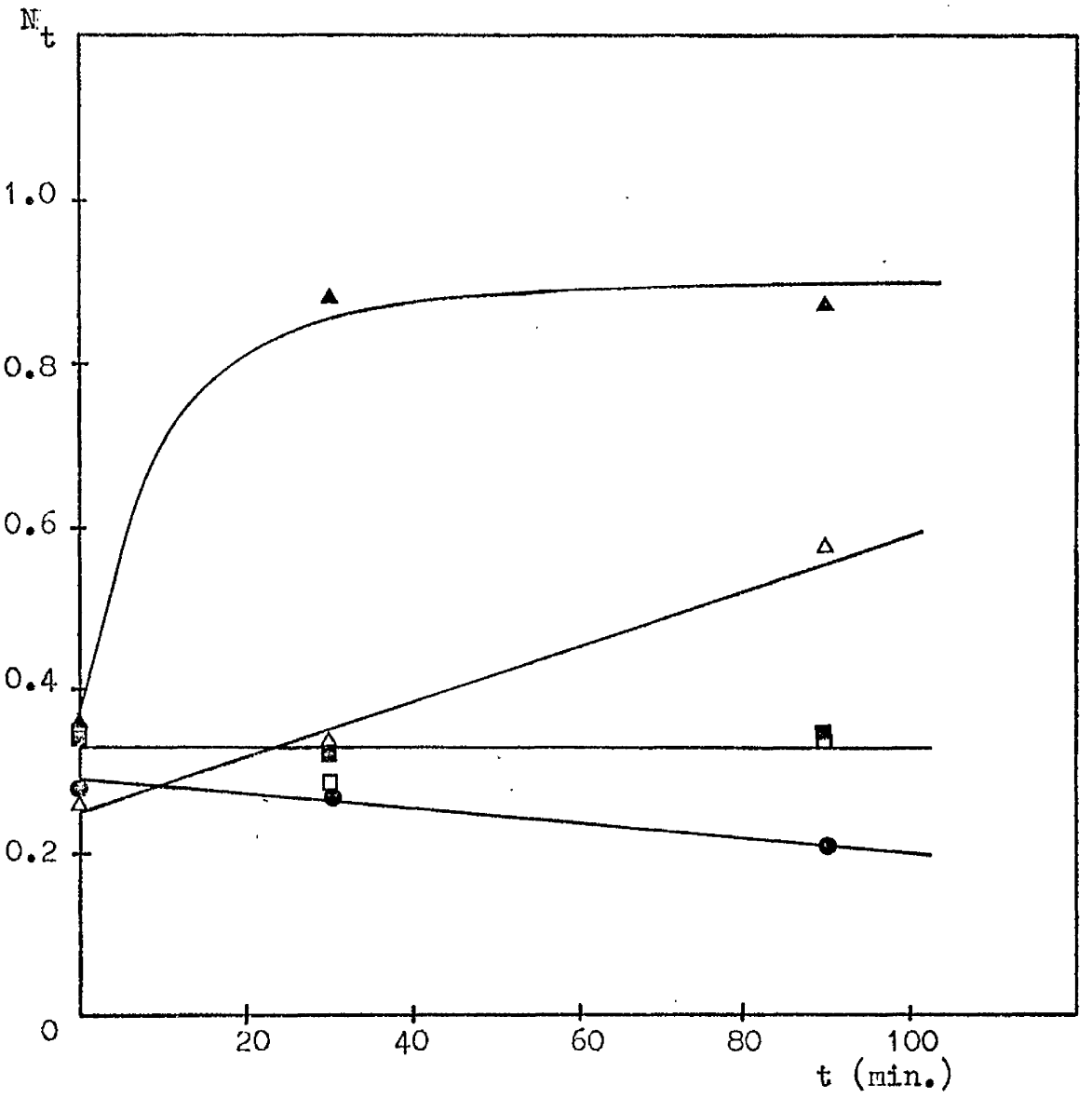


Figure 19. PHOSPHOLIPASE C TREATMENT OF C-13 PRE-FORMED AGGREGATES, exp. 13:

abscissa - as in fig. 18,
ordinate - total particles counted electronically,

flask:	control	trypsin		phospholipase C	
	●	△	▲	□	■
concentration: (μg/ml.)	Hanks'	0.18	9.0	0.2	0.5

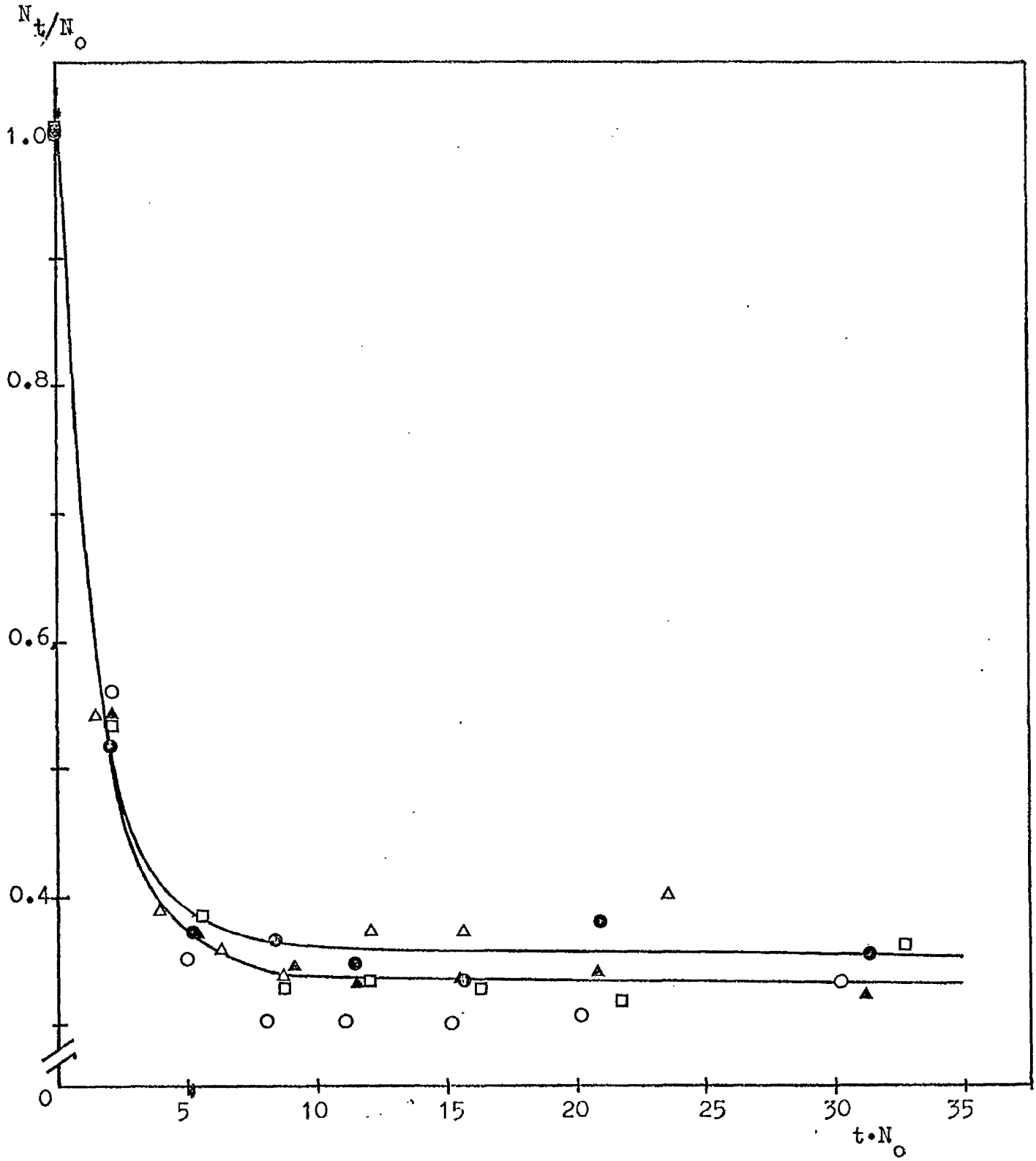


Figure 20. EFFECT OF EDTA ON C-13 AGGREGATION, exp. 14:

flask:	control		EDTA		
	●	△	○	□	▲
concentration:	Hanks'	"tris"	0.001M	0.005M	0.01M

upper curve: hanks,
lower curve: 0.01M EDTA.

THE EFFECT OF EDTA ON C-13 AGGREGATION

The cells were aggregated in either Hanks or tris with various concentrations of EDTA as indicated. See text for details. The results of a test for significance are also given.

EXPERIMENT 14 Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
8	1.0427 ±0.0175	0.6172 ±0.0118	0.6436 ±0.0060	0.6451 ±0.0327	0.0003039	0.0174

Tris;

8	0.7823 ±0.0168	0.8280 ±0.0186	0.6476 ±0.0042	0.6356 ±0.0233	0.0002806	0.0168

0.001M EDTA;

8	1.0045 ±0.0148	0.5120 ±0.0103	0.5143 ±0.0071	0.6970 ±0.0277	0.0002183	0.0148

0.005M EDTA;

8	1.0869 ±0.0192	0.5497 ±0.0121	0.5974 ±0.0079	0.6683 ±0.0381	0.0003652	0.0191

0.01M EDTA;

8	1.0340 ±0.0069	0.5577 ±0.0046	0.5767 ±0.0028	0.6696 ±0.0131	0.0000478	0.0069

DIFFERENCE:

insignificant - $\phi_{\text{Hanks}} - \phi_{\text{0.01M EDTA}}$: $t = -0.696$ (DF = 10); $p < 0.01\%$, one tail

significant - $A_{\text{Hanks}} - A_{\text{0.01M EDTA}}$: $t = 4.764$ (DF = 10); $25\% < p < 40\%$ two tails

concentration of EDTA had no detectable effect on C-13 aggregation.

F. Discussion (exps.8-14)

The main conclusion to be drawn from the results of these experiments is that protein (but not collagen) is important to intercellular adhesion in this system. A second point, in view of the insensitivity of aggregation to EDTA, is that if divalent cations are required for the adhesion, their binding to the cell surface must be of a strength for which there would appear to be no biological precedent.

The function of cell surface protein in C-13 interaction is not known but there are at least three possibilities: a) as an intercellular cement, b) complementarity to another type of molecule on an opposite cell surface,and c) as negatively charged glycoprotein providing much of the potential of the cell a) The existence of a soluble "cement", operating as Humphreys (1963)has described in sponges, can be rejected since C-13 cells aggregate rapidly in Hanks medium without addition of cellular extracts and apparently without synthesis of cementing molecules. This letter point is evident since the final extent (ϕ) of aggregation depends on only the initial cell density and not time. A "cement" firmly bound to one of the cells could not be distinguished from possibility b.

b) As a complementary molecule, protein may function as in the yeast mating strains, where a glycoprotein on one gamete binds a different glycoprotein on the opposite gamete (Brock 1958). Protein may also link cells as suggested in chick embryonic neural retinal cells by Roseman (1970). In these cells, a glycosyl transferase on one cell binds a specific sugar acceptor on another cell. However, other protein complementary structures are feasible.

c) Proteases cleave many negatively charged glycopeptides from the cell surface (see discussion previously). Their loss, according to the lyophobic colloid theory of cell adhesion (Curtis 1960), should cause a decrease in the cell surface net negative charge and repulsive force and thus cause an increase in adhesion. Cook et al (1961) have shown that trypsinization of erythrocytes greatly reduces the cell surface charge as measured by electrophoretic mobility. However, heavier treatments with trypsin cause a decrease in cell aggregation as shown above. This is probably due to the destruction of cell surface adhesive molecules but the possibility remains that progressively increasing trypsin treatment reveals more and more charged groups.

The second conclusion concerns the observations with phl C and EDTA. Lesseps (1967) found that a trypsin insensitive lanthanum staining layer on the surface of embryonic chick cells /

cells was attacked and removed by phl C. The observation that phl C failed to disperse aggregates may mean that this layer may have no adhesive properties. Calcium and other divalent cations do not appear to be of importance in C-13 aggregation. The cement and calcium bridge theories of cell adhesion predict that these ions are bound to molecules on the cell surface. However, as mentioned previously (see section on Mechanisms of Adhesion) the only known cell surface molecular group which may bind divalent cations with any considerable strength are phosphates in phospholipids (see Shah and Schulman 1967). Although phl C removes most of the cell surface phosphate groups, it has no detectable effect on cell adhesion.

The failure of EDTA to decrease aggregation of C-13 cells fails to support either the calcium bridge or lyophobic colloid theories of cell adhesion(unless the cells adhere in th primary minimum). It is conceivable that calcium sequestered by EDTA is replaced by intracellular calcium, but the concentration of calcium associated with the cell is probably far too low. For exsmple, it has been found to be 4.7 mM (for HeLa cells) and only 10% of that figure is located intracellularly (Borle 1968; also see Loewenstein 1968). The total amount of calcium in 10^6 cells in one millilitar would be several orders of magnitude less than the amounts of EDTA used in this experiment. The ineffectiveness of EDTA might be due to a cell surface /

surface molecular group binding calcium with greater strength than the chelating reagent. However, no such molecule is known.

EXPERIMENTAL III - NEURAMINIDASE

A. Introduction

1) Functions of cell surface neuraminic acid

NANA has been thought to be of importance in cellular adhesive interactions because of its major contribution to the cell surface net negative charge (Curtis 1960) and because it may be a site of calcium bridging between cells (Steinberg 1962) The functions of the neuraminic acid in regard to cellular interactions with antibodies, agglutinins and viruses have been discussed previously.

Despite these functions the physiological importance (if any) of cell surface NANA groups is not known. However, Glick et al (1965) have found that the treatment of L1210 cells with neuraminidase (NANase) decreased the potassium released by the cells in potassium-free media. The treatment also reduced the secretion of several intracellular proteins without effect on other components, i.e. glucose, sodium and lysine (Glick et al 1966).

2) Structure of cell surface NANA

NANA occurs on the cell surface only as the terminal residue(s) of species of glycolipids and glycoproteins (Blix 1959, Warren 1966, Roseman 1970). The number of these species is limited (Winzler et al 1967, Meezan et al 1969). NANA is connected by an α -ketosidic bond from its C-2 position to either /

either D-galactose, or N-acetyl-D-galactosamine (Gottschalk 1966, Roseman, 1970). Forrester et al (1962) found that the net negative surface charge of C-13 cells was due largely to NANA groups. The carboxyl group of the sugar is strongly acidic with a pK_a of $\sim$ 2.7 (Svennerholm 1957).

Neuraminic acid exists widely in nature, but most commonly as N-acetyl-neuraminic acid. However, O-acetyl and N-glycolyl groups (not known in any other biological molecule, Blix 1959) are sometimes found, (Warren 1966).

3) Clostridium perfringens NANase

Cassidy et al (1965) have compared the properties of the enzyme derived from Cl.perfringens with that from Vibrio cholera. Cl.perfringens NANase has no metal requirement unlike the V. cholera enzyme which is activated by Ca^{++}; but otherwise both have similar activities. There is no difference in their specificities, which include all the neuraminic acids (Uefelson et al 1966). The pH optimum is between 5 and 6 but the enzymes are also active at pH 7 (Ada et al 1961).

Cassidy et al (1965) also found that the C3 and C6 isomers of NANA-galactose were cleaved at low rates. Some neuraminic acid residues in porcine and bovine submaxillary mucins and calf brain ganglioside were resistent to NANase.

Except /

Except where stated, Sigma NANase[27], batch number 99B-8100, was used in each experiment. Disc electrophoresis of the enzyme on 7.5 and 15% acrylamide gels showed that the preparation contained at least four proteins of molecular weight $\sim 10^4$ and one protein of molecular weight $\sim 3 \times 10^5$ (see Appendix II, fig.35).

B. Fluorimetric Assay of NANase Activity

1) Introduction

It is necessary to examine whether or not the enzyme is active in removing NANA from the cell surface. All too often, this has not been done with NANase preparations. The assay was modified from that developed by Comb and Roseman (1963). This particular assay was used because of its unequalled specificity and sensitivity for the most common neuraminic acids (Brunetti et al 1963, and see Eickberg and Karnovsky 1966).

a) General description of the assay

The effectiveness of NANase was measured on cells cultured in plastic petri dishes at pH 7.2, thus the damaging effects of lower pH's on the cells were avoided. After various times of incubation an aliquot of NANase containing cell supernatant was withdrawn and treated with neuraminic acid aldolase. The concentration of pyruvate, produced from NANA by the /

the aldolase, was measured by its conversion to lactate, using a lactate dehydrogenase-NADH system. The decrease in NADH was measured fluorimetrically.

b) Neuraminic acid aldolase

This enzyme cleaves NANA into pyruvic acid and N-acetylmannosamine (Rafelson et al, 1966). N-glycol-neuraminate is cleaved at a rate 65% of that of NANA and N-O-diacetyl neuraminate at 14% of NANA. The other neuraminic acids are not attacked. The enzyme also forms NANA from pyruvate and N-acetyl-mannosamine substrates with an equilibrium constant of 0.064 M at 37°C (Comb and Roseman 1962)

The pH optimum is 7.2 but the enzyme is effective at 6.8 - 8.0. Aldolase preparations are often found to be contaminated with NADH oxidase (Comb and Roseman 1962).

2) Methods

a) The essay

a) C-13 cells were plated into 60 mm. diameter plastic petri dishes[28] at 10^6 cells/dish and grown for two days in 4 mls. of 811 until confluent. b) The cultures were gently washed in 100 mls. of warm Hanks once and then given three washes in 100 mls. of warm glucose-free Hanks (GFH)[29]. Each culture was then incubated in two changes of GFH for approximately one hour. The purpose of the GFH incubation was to help reduce the /

the considerable amount of pyruvate secreted by the cells during the assay (see fig.24). All operations were at 37°C and the cultures were handled gently to attempt to reduce damage to the monolayer. c) After GFH incubation the cultures were washed twice in GFH and then thoroughly drained (drying was avoided). Subsequently, one ml. of NANase in GFH was added to each dish, at the concentrations indicated. d) The cultures were incubated in a moist container at 37°C. At various times, they were withdrawn and aliquots of 0.9 mls. were removed from each culture and added to a test tube containing either 0.1 ml. of 0.02 M phosphate buffer[30], pH 7.2 (in duplicate to measure pyruvate released from cells) or 0.1 ml. of buffer with 0.056 units aldolase[31] (to determine the NANA released). e) Each solution was mixed and incubated in a 37°C water bath for 60 min in sealed test tubes. f) After this time, the tubes were stoppered with Kjeldahl caps and immersed in a boiling water bath for two minutes to destroy the NADH oxidase found in this aldolase and to precipitate the protein. The solutions were then placed in an ice bath. g) At this point, 0.9 ml. of each sample was removed to another test tube and mixed with 0.1 ml. of LDH-NADH in GFH (final concentrations: LDH[32], 0.002 mg/ml; NADH[33], 1.024×10^{-2} M/ml). h) This solution was then micropore filtered using 0.3 μ filters[34] to remove precipitated protein. Therefore the samples /

samples differed only in their pyruvate, neuraminate and aldolase content.

b) Fluorimetry

The solutions were transferred to small cups and sampled automatically at the rate of four/minute. Approximately 0.8 ml. of each solution was passed through the flow cell of a Locarte MK-5 fluorimeter[35] with a primary LF-2 filter and a secondary LF-5 filter. Between each sample were one GFH blank and two water blanks.

The pen recorder was calibrated by measuring the difference in fluorescence between the GFH blanks and controls containing known concentrations of NADH in GFH.

3) Controls

a) Pyruvic acid assay

The efficiency of the LDH-NADH system was examined in conjunction with the assay for cellular NANA. Different concentrations of pyruvate[36] in GFH (0.9 mls., 0.5 - 5.0 x 10^{-3} μM/ml.) were mixed with 0.1 ml. of LDH-NADH as above and incubated at room temperature.

The results for two experiments are shown in fig.21 and indicate a linear relationship between the amount of pyruvate added and the amount of NADH destroyed.

b) NANA assay

The activity of the aldolase was tested by coupling the products of an aldolase-NANA reaction to the LDH-NADH system./

system. This was also done in conjunction with assays for cellular NANA.

NANA[37] was dissolved in GFH at concentrations ranging from 1.0 - 5.0 x 10^{-3} M/ml. As before, 0.9 mls. of NANA were mixed with 0.1 mls. of aldolase and placed in a 37°C water bath for 60 mins. The solutions were then boiled for 2 mins. to destroy NADH oxidase, cooled, and then 0.9 mls. was transferred to a test tube and mixed with 0.1 ml. of LDH-NADH solution. The samples were micropore filtered as before and incubated at room temperature before being read. The results are shown in fig.22.

c) NADH decay

NADH was made up at the beginning of each experiment, immediately before fluorimetry. During the sampling period, the fluorescence of the standard NADH decreased as seen in fig. 23. The effect of this decay was taken into account and corrected for each sample.

4) Results

The total amount of pyruvate measured in each culture and that due to NANA is shown in fig.24. Approximately 3 x 10^{-3} M NANA/10^{6} cells is released by NANase but the amount is a function of enzyme concentration. This value is more than twice that reported for C-13 cells by Kraemer (1967) and over 400-fold greater than that reported for chick embryonic cells /

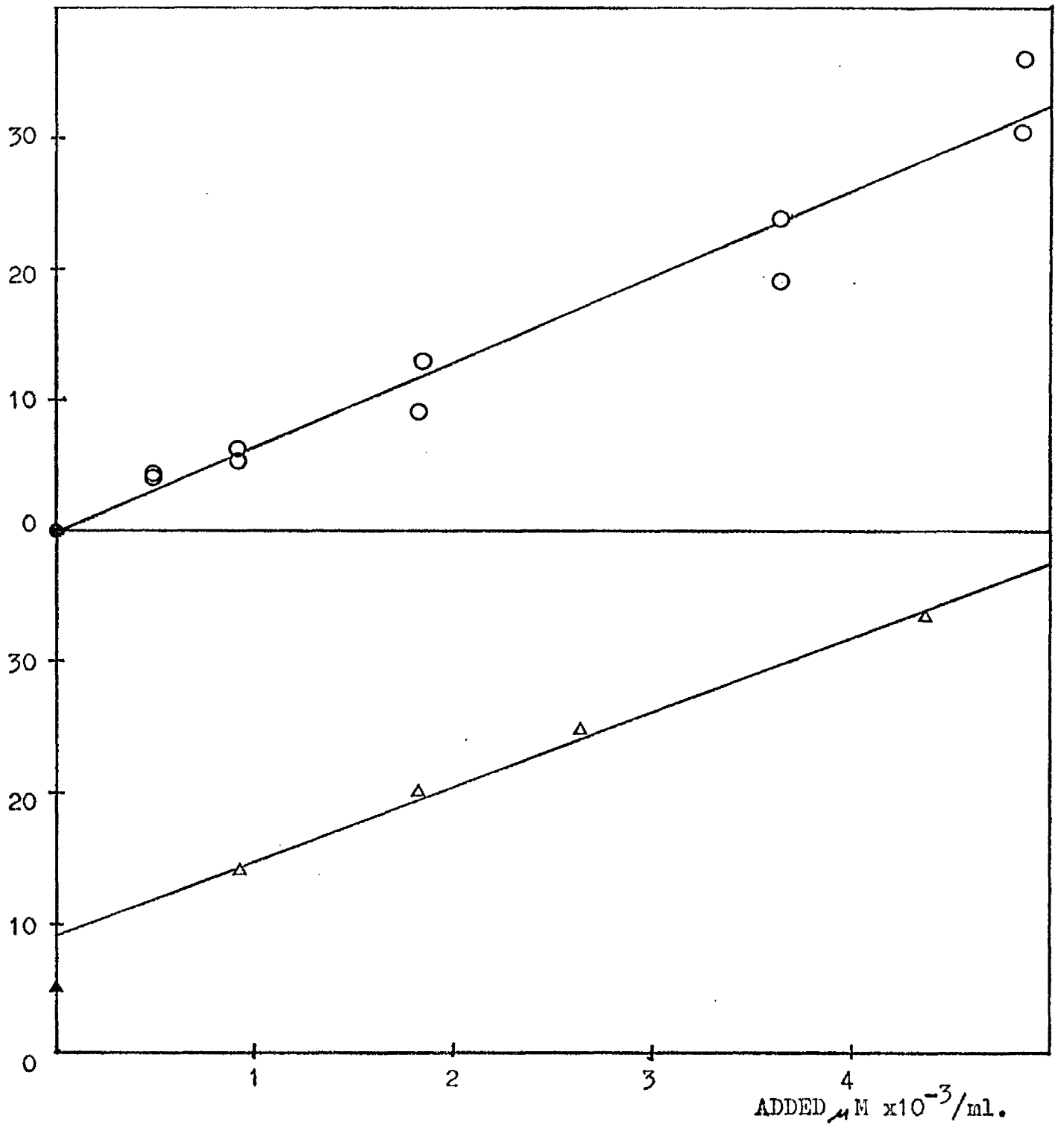


Figure 21 above. CONTROL ASSAY FOR PYRUVIC ACID:
abscissa - concentration of pyruvate added to the system,
slope = 0.154×10^{-3} μM/unit; r = 0.982; slope $\frac{\text{NADH used}}{\text{pyr. added}} = 0.90$

Figure 22 below. CONTROL ASSAY FOR NANA:

abscissa - concentration of added NANA, slope = 0.102×10^{-3} μM/unit;
r = 0.998.

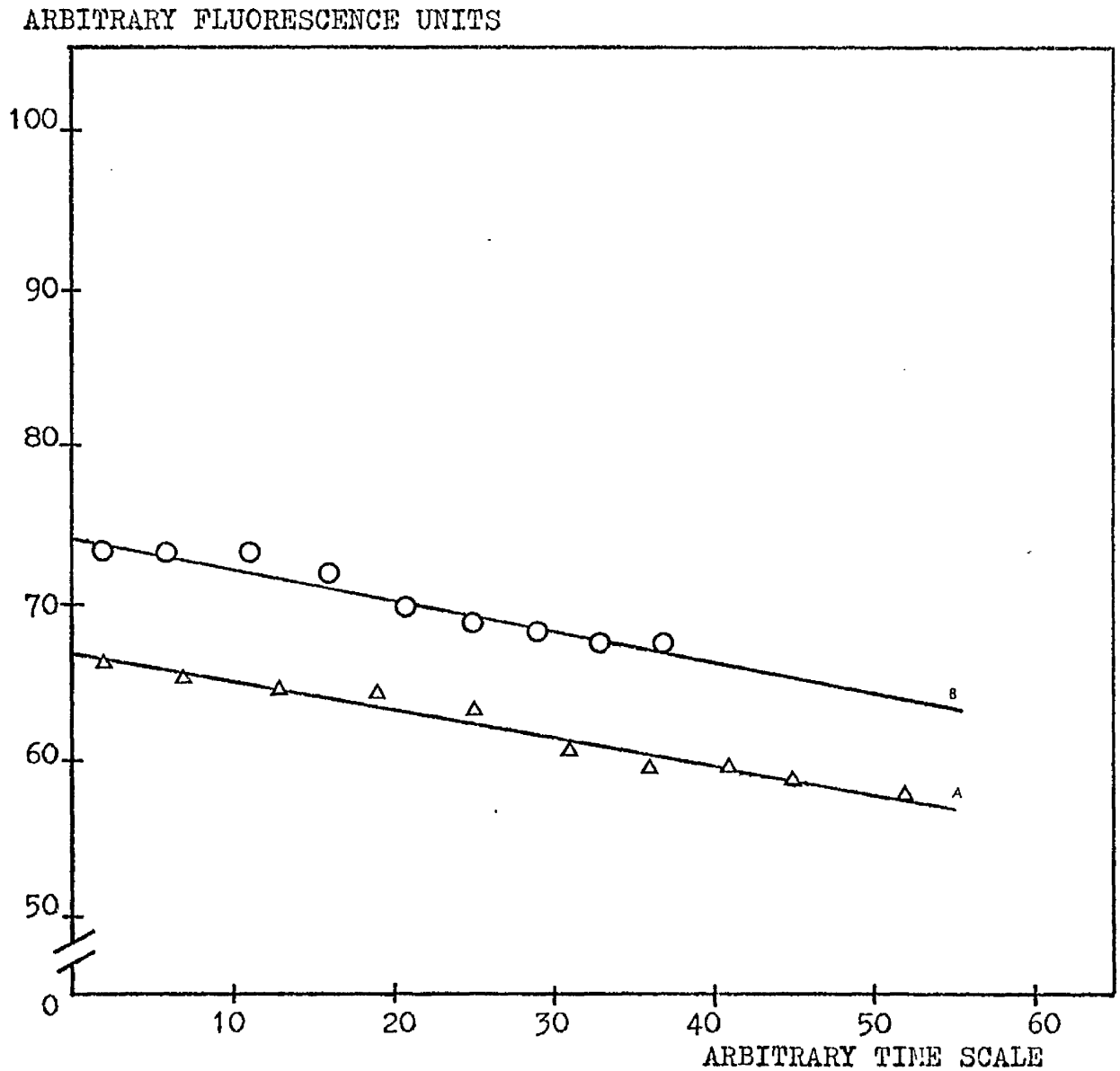


Figure 23. NADH DECAY:

Regression of the loss of fluorescence of the NADH solutions during the assays for NANA,

exp.	slope	S_b
A	-0.182/	0.011
B	-0.198/	0.020

per time unit

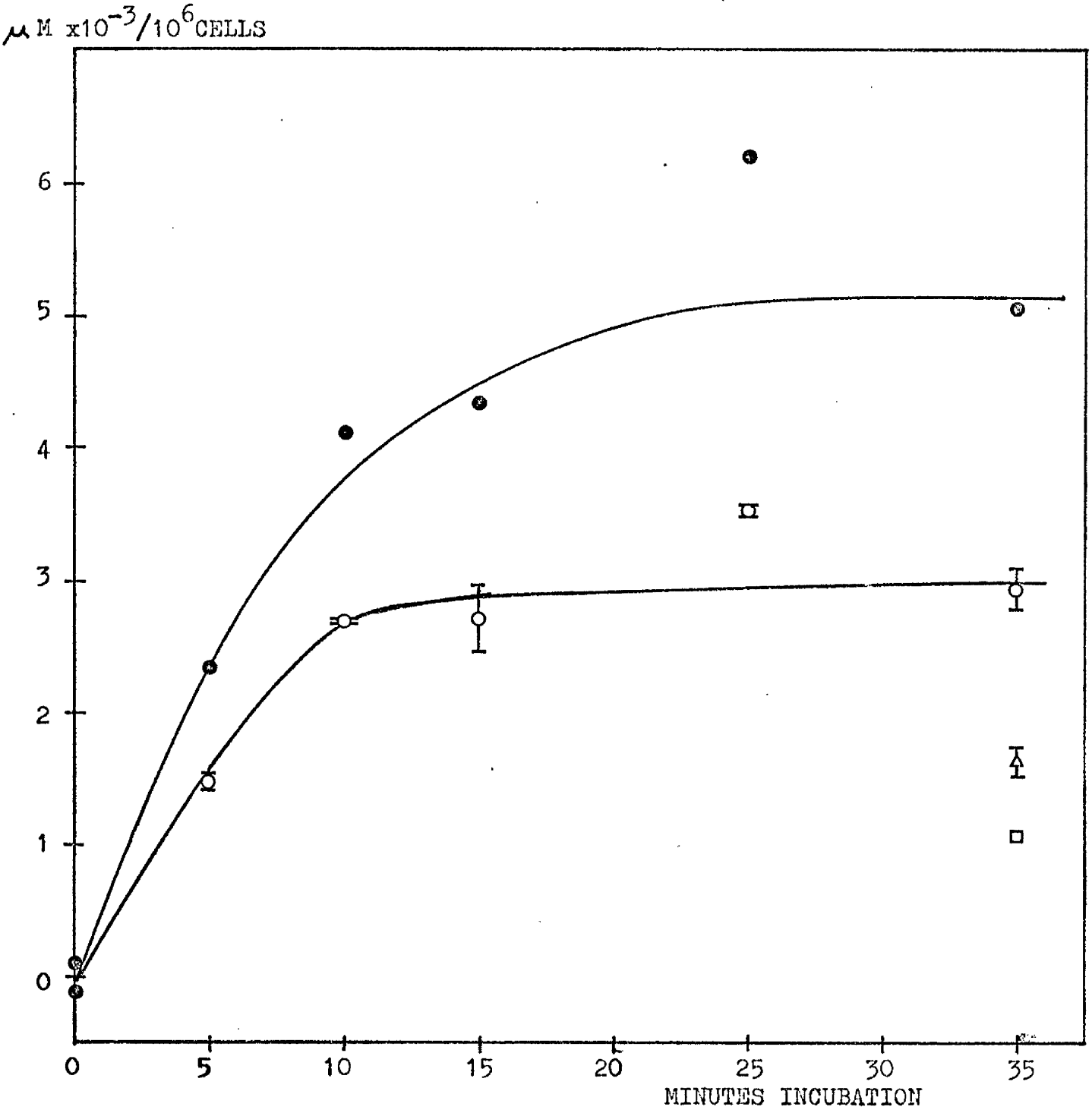


Figure 24. CELL SURFACE NANA RELEASED BY NANASE TREATMENT:

abscissa - time of enzyme incubation with the cell monolayer
ordinate - equivalent amount of pyruvic acid standard

1) ● total pyruvate released from cells treated with NANase and aldolase,
2) ○
3) △ } pyruvate derived from NANA alone (curve 1, minus pyruvate released
4) □ } from cells not treated with ~~NANase~~ aldolse).

NANase concentration: (mg/ml)	○ 0.05	△ 0.025	□ 0.005

cells by Kemp (1970). Comparison of these results with my own is difficult since both authors first disaggregated the cells with extensive trypsin treatments before applying NANase. Kemp (1970) found that this initial trypsin treatment of chick cells removed 2.8×10^{-3} μM NANA/10^6 cells and subsequent NANase treatment released only 1.3×10^{-5} μM NANA/10^6 cells.

C. NANase and C-13 aggregation

To examine the possible function of cell surface NANA in intercellular adhesion cells were treated with NANase in either one of three ways: (i) addition of the enzyme to stable aggregates, (ii) enzyme addition at t = o, as aggregation begins as done by Kemp (1968), and (iii) pre-treatment of cultures with the enzyme before testing aggregation.

1) NANase on Aggregates

a) Methods

In a series of 5 experiments (exps.III 1 - 5) NANase was added to completely aggregated C-13 suspensions in shakers, at 37°C. The suspensions were first aggregated for 30 - 50 minutes in Hanks, until the system had become stable, and then 0.1 mls. of NANase in Hanks was added. In all but the first experiment, the NANase used was from the same batch as that used in the enzyme assay.

b) Results

In/

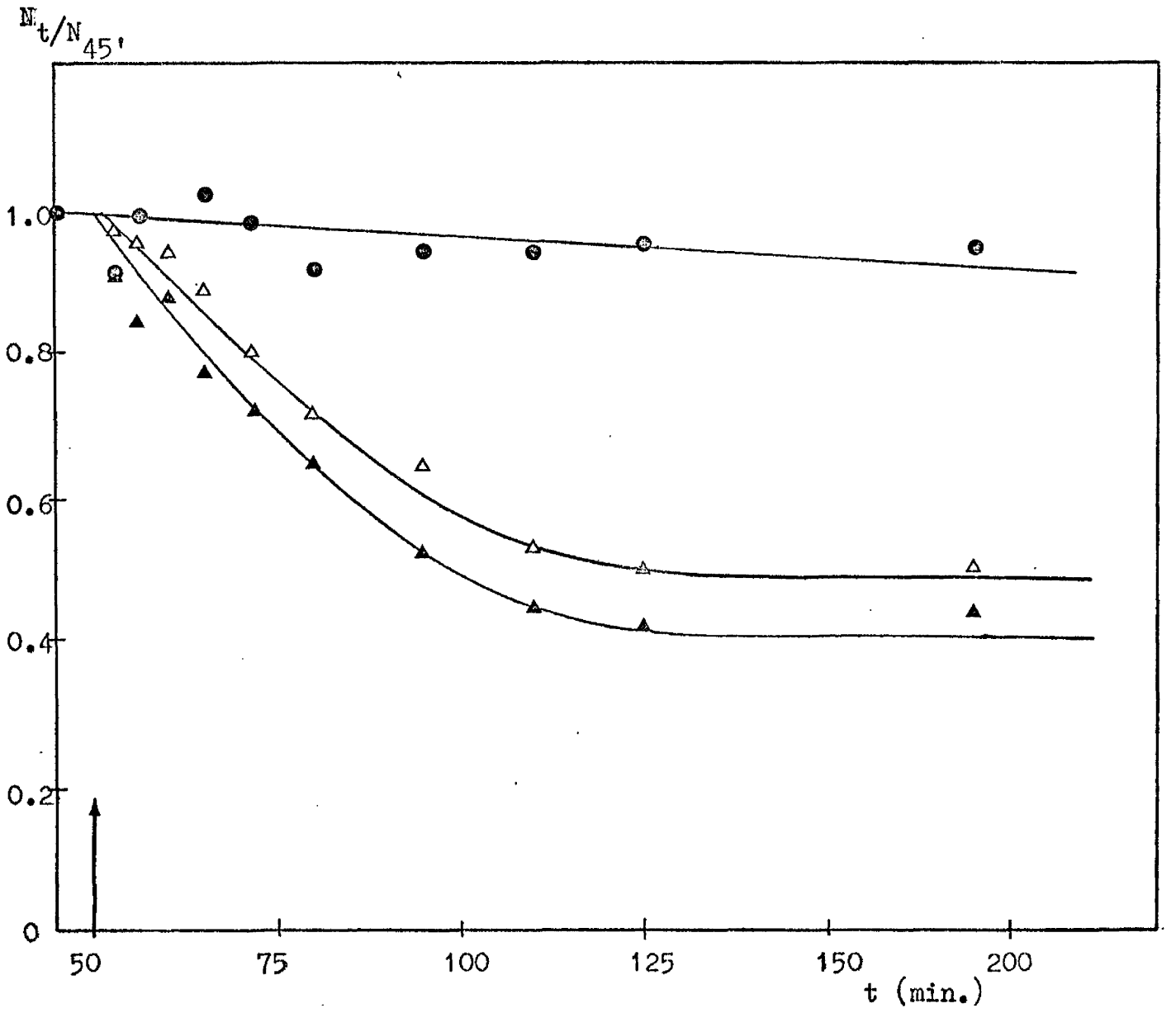


Figure 25. TREATMENT OF PRE-FORMED C-13 AGGREGATES WITH NANASE, exp. 3:
abscissa - time during aggregation; arrow indicates addition of NANase,
ordinate - relative increase in aggregation compared with the particle concentration before addition of NANase, (δ),
● Hanks', △ NANase 0.01mg/ml., ▲ NANase 0.05mg/ml.

experiment:	δ/Hanks' control,	δ/NANase treated (with NANase concentration)
1	1.042 (pairs)	0.798 (pairs) (0.051mg/ml.)
2	1.033	0.868(0.05mg/ml); 0.786(0.025mg/ml); 0.871(0.005mg/ml)
3	0.949	0.505(0.01mg/ml); 0.444(0.05mg/ml)
4	1.006	0.521 " " ; 0.584 " "
5	0.881	0.550(0.025mg/ml).

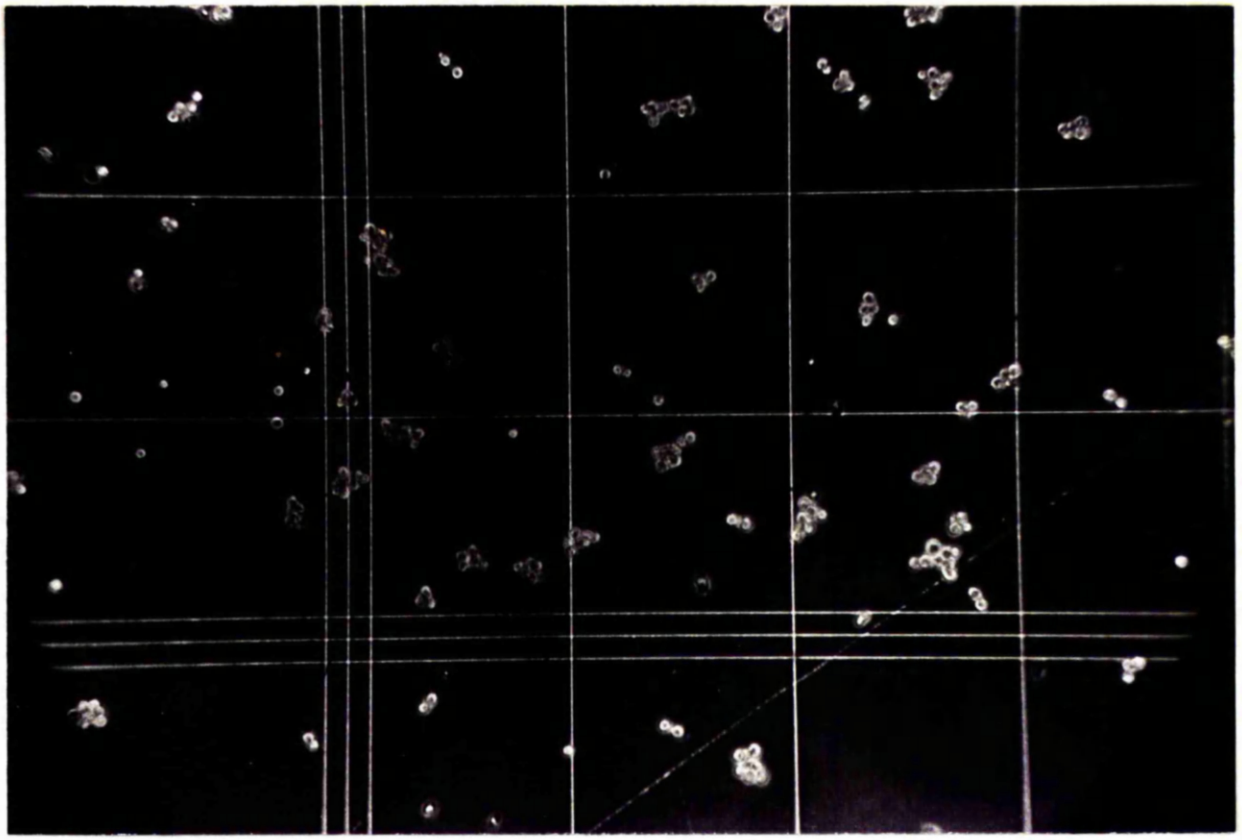
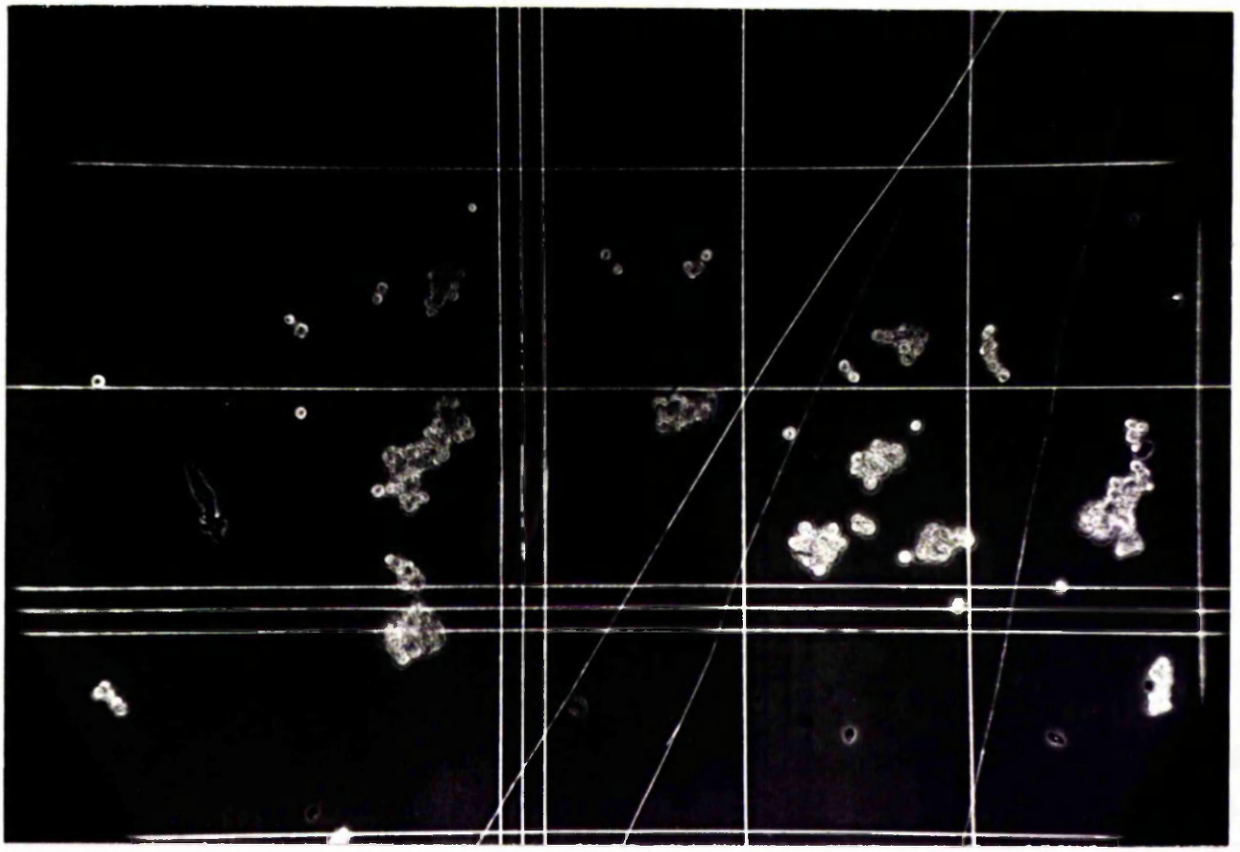


fig. 27. NANase treated suspension after 130'. 0.025mg/ml. NANase, $\emptyset$ = 0.205.



Darkfield illumination,

Magnification: x284

In all but exp.1 NANase caused an immediate increase in aggregation involving all particle size classes as a function of enzyme concentration (fig.25). The additional aggregation was substantial with Ø being increased by about 50% above aggregation without NANase. The size of the aggregates in the NANase suspensions was doubled compared with those in the control suspensions (figs. 26,27).

2) NANase pre-treatment of cultures: enzyme concentration

By exposing cells to NANase, and then washing the enzyme away before cell aggregation any non-enzymic effects which the enzyme containing media may have on cell adhesion are likely to be eliminated unless some molecules in the enzyme solution bind irreversibly to the cell surface.

a) Methods

In six separate experiments (exps.6-11) C-13 cells were grown in four 75cm^2 plastic bottles for two days until confluent or nearly confluent. The cultures were first washed several times in warm Hanks' and then incubated at 37^{o}C in various concentrations of NANase dissolved in Hanks' for the times indicated. After incubation the NANase was removed and the cultures were washed one to three times in tris at room temperature.

Each culture was then treated with 0.05% trypsin-EDTA for /

for 3-4 minutes at room temperature, harvested and washed twice each in tris and Hanks' at 0°C. (The longer trypsin incubation times were sometimes necessary for harvesting from the plastic).

The cells were resuspended in Hanks at $\sim 10^6$/ml. and were composed of almost 100% single cells with up to 15% non-phasing cells. NANase did not appear to have any deleterious effect on cell viability. Most of the non-phasing cells recovered upon incubation at 37°C.

In one experiment NANase was added to a previously untreated suspension at t = o (final concentration 0.15 mg/ml).

b) Results

In all but one of the experiments NANase pre-treatment caused an increase in the value of $\emptyset$ compared to the control. The effect seemed greater if less densely grown cells were treated, and if the treatment was not prolonged. This latter point could indicate the existence of a contaminant in the NANase which has a deleterious effect on cell aggregation (see fig.28 and table 4).

Microscope observations of experiments 6 and 11 confirmed that after 40 minutes of aggregation the clusters in the enzyme pre-treated suspensions were larger than those in the controls. There seemed no detectable effect of NANase on the aggregation rate constant A.

3) /

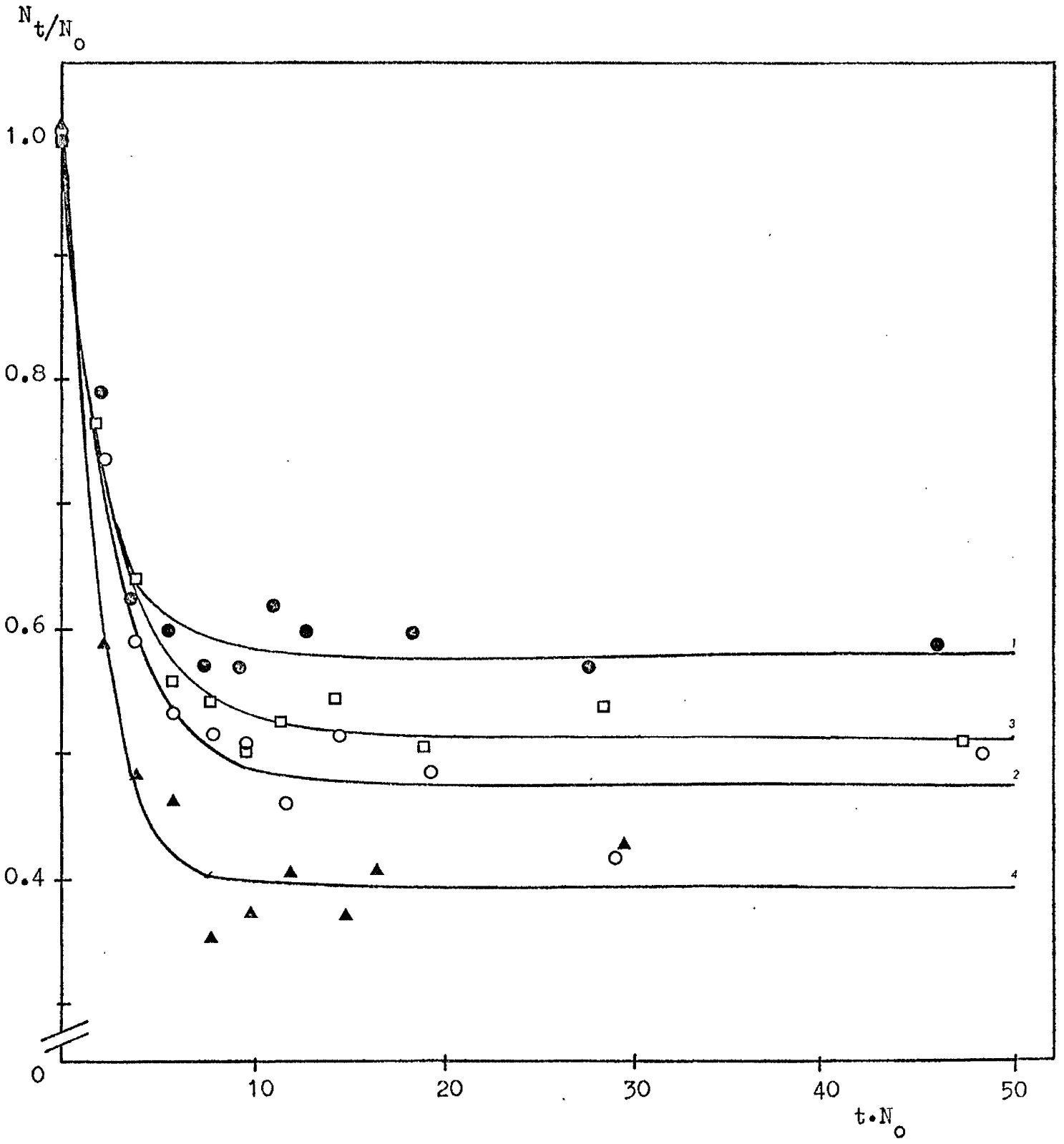


Figure 28a. PRE-TREATMENT OF C-13 CULTURES WITH VARIOUS CONCENTRATIONS OF NANASE BEFORE AGGREGATION, exp. 10:

Hanks', control	NANase (pre-treatment)		NANase (added at t = 0)	
1 ●	2 ○	0.01mg/ml.	4 ▲	0.05mg/ml.
	3 □	0.05 "		

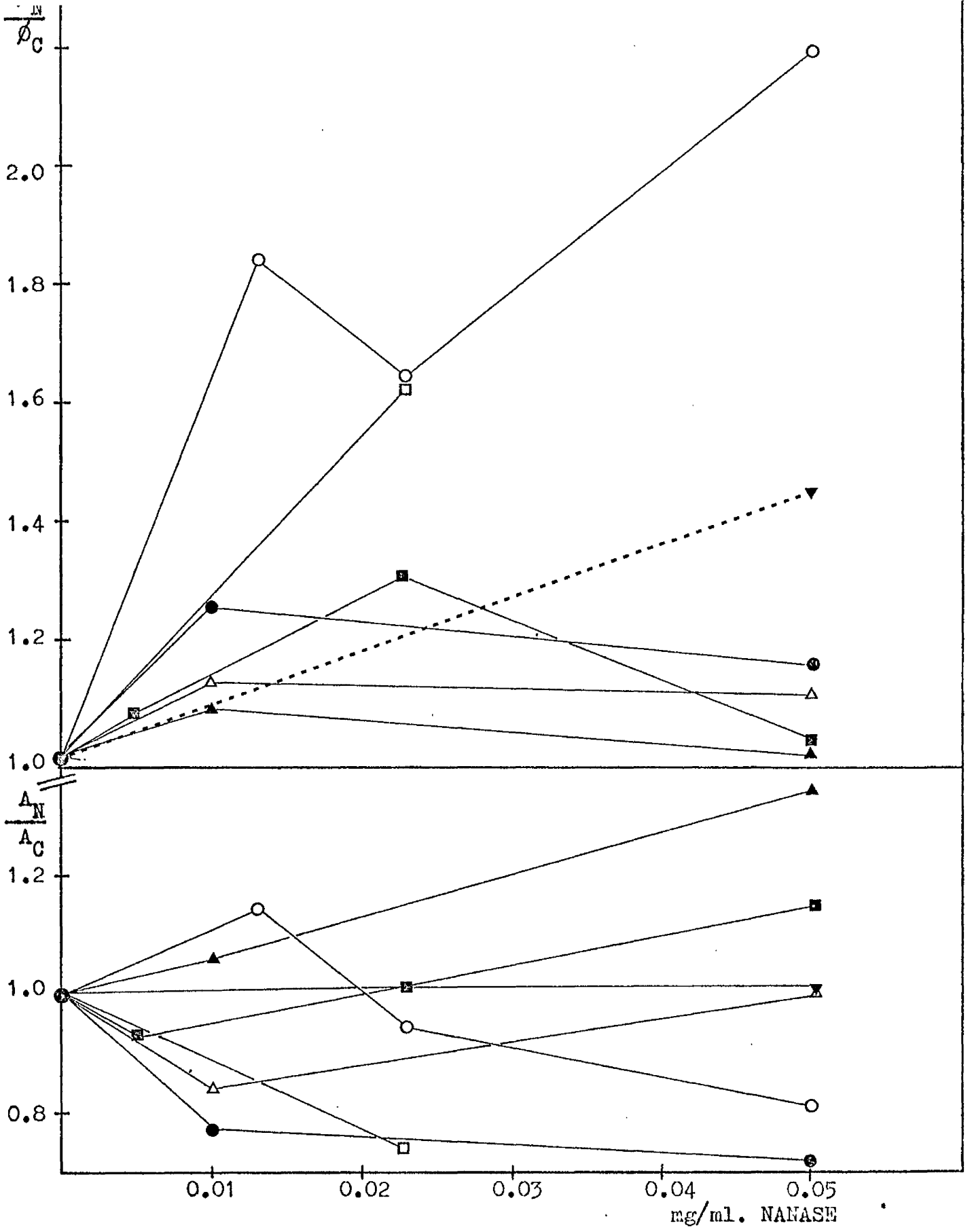


Figure 28b. PRE-TREATMENT OF C-13 CULTURES WITH VARIOUS CONCENTRATIONS OF NANASE BEFORE AGGREGATION:
Relative aggregation compared to that in the controls:
abscissa - concentration of NANase used,
ordinate - parameters of the NANase-treated (N) suspensions divided by that of the control (C).
experiment: 6 ■; 7 □; 8 ▲; 9 △; 10 ●; 11 ○;
▼ NANase added at t = 0.

TABLE 4

PRE-TREATMENT OF C-13 CULTURES WITH VARIOUS CONCENTRATIONS OF NANASE, and the addition of the enzyme at the start of aggregation

The concentration of NANase and the time of pre-incubation (minutes at 37°C) are given. See text for details. Most of the cultures were harvested from dense, confluent monolayers; but in exp. 10 the growth density was ~80% and in exp. 11 it was ~50%. In some cases, where the significance of the difference in the values of ϕ between treatments is in doubt, the results of t tests are included.

EXPERIMENT 6: 30 min. incubation;

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
8	0.9663 ±0.0295	0.1315 ±0.0213	0.1271 ±0.0202	0.2704 ±0.0264	0.0006498	0.0255

0.005mg/ml. NANase;

8	0.9945 ±0.0409	0.1218 ±0.0325	0.1212 ±0.0320	0.2918 ±0.0371	0.0012253	0.0350

0.025mg/ml. NANase;

8	1.0174 ±0.0334	0.1331 ±0.0296	0.1354 ±0.0297	0.3511 ±0.0335	0.0008445	0.0291

0.05mg/ml. NANase;

8	1.0296 ±0.0318	0.1528 ±0.0203	0.1574 ±0.0203	0.2794 ±0.0329	0.0008005	0.0283

significant difference, $\phi_{\text{Hanks}} - \phi_{0.025\text{mg/ml. NANase}}$: $t = -1.906$ (DF = 10), $5.0\% > p > 2.5\%$

TABLE Continued/

TABLE 4 Continued

EXPERIMENT 7: 25 min. incubation;

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
8	1.0458 ±0.0372	0.1832 ±0.0146	0.1916 ±0.0137	0.1927 ±0.0388	0.0011647	0.0341

0.025mg/ml. NANase;

8	0.9940 ±0.0195	0.1360 ±0.0146	0.1352 ±0.0142	0.3125 ±0.0181	0.0002924	0.0171

EXPERIMENT 8: 30 min. incubation;

control, Hanks;

12	0.9114 ±0.0223	0.2225 ±0.0209	0.2028 ±0.0184	0.4379 ±0.0300	0.0004185	0.0205

0.01mg/ml. NANase;

12	0.8974 ±0.0228	0.2360 ±0.0222	0.2117 ±0.0192	0.4738 ±0.0316	0.0004440	0.0211

0.05mg/ml. NANase;

11	0.8169 ±0.0253	0.2986 ±0.0213	0.2439 ±0.0157	0.4583 ±0.0310	0.0005666	0.0238

EXPERIMENT 9: 30 min. incubation;

control, Hanks;

11	1.1128 ±0.0148	0.2149 ±0.0141	0.2392 ±0.0153	0.5503 ±0.0272	0.0001922	0.0139

0.01mg/ml. NANase;

10	1.0463 ±0.0151	0.1811 ±0.0194	0.1894 ±0.0201	0.6204 ±0.0247	0.0001887	0.0137

0.05mg/ml. NANase;

10	1.0802 ±0.0257	0.2106 ±0.0255	0.2275 ±0.0271	0.6133 ±0.0423	0.0004923	0.0222

insignificant difference, $\phi_{\text{Hanks}} - \phi_{0.05\text{mg/ml. NANase}}$: $t = -1.264$ (DF = 15), $p > 10\%$ one tail

significant difference, " $- \phi_{0.01\text{mg/ml. NANase}}$: $t = -1.909$ (DF = 15), $5.0\% > p > 2.5\%$ one tail

TABLE CONTINUED/

TABLE 4 Continued

EXPERIMENT 10: 30 min. incubation & at t = 0;

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
11	0.9197 ±0.0239	0.4851 ±0.0156	0.4462 ±0.0084	0.4179 ±0.0320	0.0005579	0.0236

0.01mg/ml. NANase;

11	0.9643 ±0.0276	0.3719 ±0.0200	0.3586 ±0.0164	0.5244 ±0.0432	0.0007249	0.0269

0.05mg/ml. NANase;

11	0.9456 ±0.0220	0.3496 ±0.0259	0.3306 ±0.0129	0.4859 ±0.0320	0.0004546	0.0213

At t = 0, 0.046mg/ml. NANase;

10	0.9824 ±0.0325	0.5344 ±0.0228	0.5250 ±0.0141	0.6061 ±0.0572	0.0010428	0.0323

significant difference, $\phi_{\text{Hanks}} - \phi_{\text{NANase at t=0}}$: t = -3.014 (DF = 15), 0.5% > p > 0.25%

" " , $\phi_{\text{0.05mg/ml. NANase}} - \phi_{\text{NANase at t=0}}$: t= -1.918(DF=15), 5.0% > p > 2.5%

EXPERIMENT 11: 5 min. incubation;

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
9	0.5174 ±0.0094	0.9369 ±0.0170	0.4848 ±0.0008	0.1975 ±0.0054	0.0000865	0.0093

0.0125mg/ml. NANase:

10	0.5581 ±0.0163	1.0655 ±0.0313	0.5946 ±0.0020	0.3626 ±0.0123	0.0002627	0.0162

0.025mg/ml. NANase;

10	0.4837 ±0.0100	0.8846 ±0.0185	0.4279 ±0.0015	0.3235 ±0.0062	0.0000970	0.0098

0.05mg/ml. NANase;

10	0.5903 ±0.0176	0.7654 ±0.0237	0.4518 ±0.0039	0.4316 ±0.0150	0.0003014	0.0174

3) NANase pre-treatment of cultures: time of incubation

a) Methods

In 7 separate experiments (exps.12-18) C-13 cells were cultured, as before, for two days in plastic bottles; but the cells were plated at a lower initial density in order that the cultures would be less dense when harvested. Again, after draining away the 811 medium, each culture was gently washed four times in warm Hanks' and then incubated at 37°C in 0.05 or 0.025 mg/ml NANase in Hanks. Incubation time was varied between cultures as indicated.

After pre-treatment the enzyme solution was removed and the cultures were washed three times in tris at room temperature The cells were then harvested and resuspended at $\sim 10^6$/ml. in Hanks at 0°C. The suspensions were composed almost totally of single, phasing cells.

b) Results

In almost all experiments increased times of NANase pre-incubation caused an increase in ϕ. The values of the rate constant A were not detectably affected. (see fig.29 and table 5).

4) Growth density

The results of the pre-treatment experiments suggested that cells grown to a low density responded more to NANase than did densely grown cells. The effect of NANase pre-treatment on aggregation as a function of culture density was examined in/

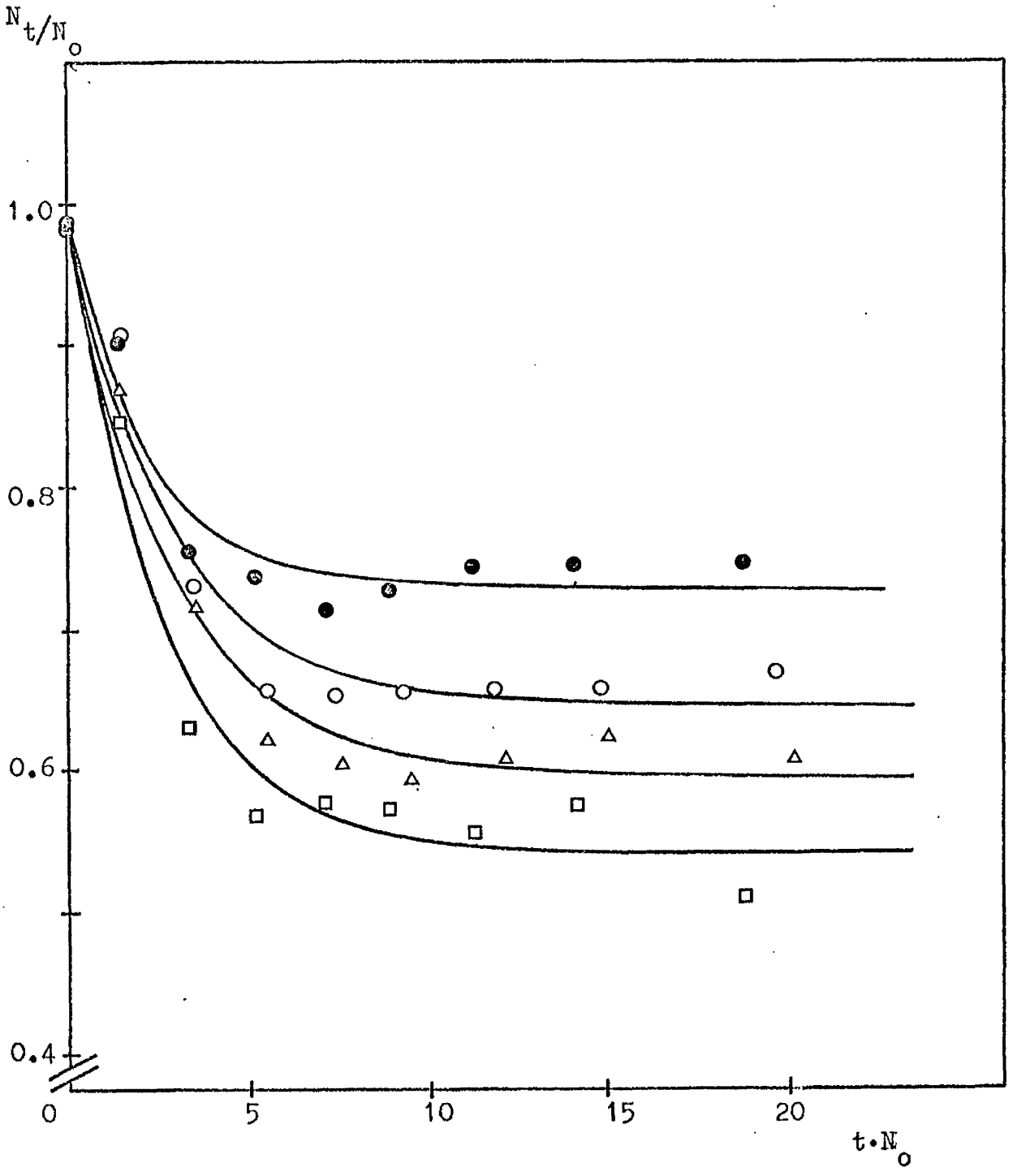


Figure 29a. PRE-TREATMENT OF C-13 CULTURES WITH NANASE FOR VARIOUS TIMES, exp. 15:

Hanks', control ● NANase, 0.025mg/ml.
○ 2' incubation before aggregation.
△ 4' "
□ 8' "

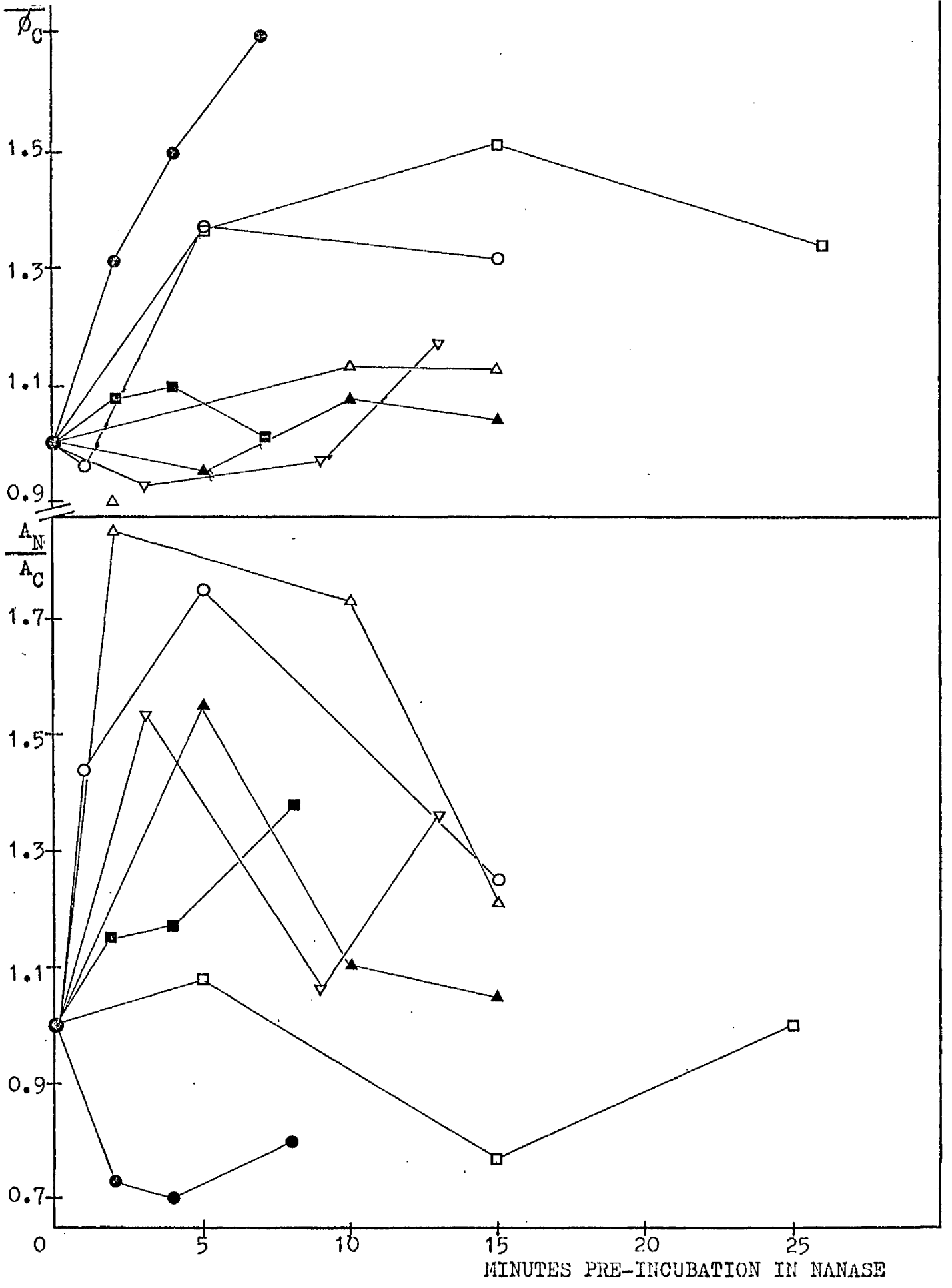


Figure 29b. PRE-TREATMENT OF C-13 CULTURES WITH NANASE FOR VARIOUS TIMES;
Relative aggregation compared to that in the controls: co-ordinates as in fig. 28,
experiment: 12 □; 13 ■; 14 ○; 15 ●; 16 △; 17 ▲; 18 ▽.

TABLE 5

PRE-TREATMENT OF C-13 CULTURES WITH NANASE FOR VARIOUS TIMES

The concentration of NANase and time of pre-incubation at 37°C are given; see text for details. The approximate growth density is also included. The significance of differences in ϕ in some marginal cases are included as before.

n	No	A	α	ϕ	Residual Variance	SEp
EXPERIMENT 12: c. 50% growth, 0.05mg/ml. NANase;						
control, 30' pre-incubation in Hanks;						
11	0.8006 ±0.0303	0.4136 ±0.0190	0.3311 ±0.0087	0.3175 ±0.0305	0.0007360	0.0271
5', NANase;						
11	0.7533 ±0.0305	0.4464 ±0.0233	0.3363 ±0.0111	0.4361 ±0.0325	0.0007479	0.0273
15', NANase;						
11	0.8320 ±0.0335	0.3181 ±0.0257	0.2646 ±0.0186	0.4858 ±0.0383	0.0008391	0.0290
25', NANase;						
11	0.8685 ±0.0207	0.4559 ±0.0137	0.3960 ±0.0072	0.4246 ±0.0258	0.0003603	0.0190
EXPERIMENT 13: c. 95% growth, 0.025mg/ml. NANase;						
control, 5' pre-incubation in Hanks;						
10	0.8047 ±0.0248	0.2668 ±0.0245	0.2147 ±0.0186	0.5421 ±0.0259	0.0004882	0.0221
2', NANase;						
10	0.7235 ±0.0363	0.3068 ±0.0388	0.2220 ±0.0257	0.5868 ±0.0355	0.0010524	0.0324
4', NANase;						
10	0.7534 ±0.0310	0.3112 ±0.0293	0.2345 ±0.0199	0.5304 ±0.0315	0.0007837	0.0280
8', NANase;						
10	0.7237 ±0.0270	0.3687 ±0.0253	0.2668 ±0.0154	0.5465 ±0.0281	0.0006141	0.0248

TABLE CONTINUED/

TABLE 5 Continued

EXPERIMENT 14: c. 50-60% growth, 0.025mg/ml. NANase;

control, 7' pre-incubation in Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
9	0.9411 ±0.0286	0.5930 ±0.0186	0.5581 ±0.0045	0.2529 ±0.0316	0.0008099	0.0285

1', NANase;

9	0.9217 ±0.0394	0.8537 ±0.0367	0.7869 ±0.0035	0.2416 ±0.0425	0.0015511	0.0394

5', NANase;

9	0.9339 ±0.0222	1.0365 ±0.0248	0.9680 ±0.0019	0.3466 ±0.0273	0.0004947	0.0222

15', NANase;

9	0.7844 ±0.0280	0.7428 ±0.0271	0.5826 ±0.0045	0.3333 ±0.0280	0.0007781	0.0279

significant difference, $\phi_{\text{Hanks}} - \phi_{15' \text{ NANase}}$: $t = -1.908$ (DF = 12), $5.0\% > p > 2.5\%$

EXPERIMENT 15: c. 70% growth, 0.025mg/ml. NANase;

control, 5' pre-incubation in Hanks;

9	0.9351 ±0.0235	0.5060 ±0.0137	0.4732 ±0.0047	0.2682 ±0.0261	0.0005275	0.0230

2', NANase;

9	0.9867 ±0.0339	0.3693 ±0.0177	0.3643 ±0.0122	0.3525 ±0.0419	0.0010517	0.0324

4', NANase;

9	1.0076 ±0.0276	0.3545 ±0.0152	0.3572 ±0.0118	0.4029 ±0.0362	0.0006936	0.0263

8', NANase;

9	0.9408 ±0.0319	0.4024 ±0.0199	0.3786 ±0.0137	0.4566 ±0.0415	0.0009372	0.0306

TABLE CONTINUED/

TABLE 5 Continued

EXPERIMENT 16: c. 80% growth, 0.025mg/ml. NANase;

control, 9' pre-incubation in Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
10	0.7254 ±0.0257	0.3661 ±0.0218	0.2656 ±0.0127	0.4458 ±0.0256	0.0005572	0.0236
2', NANase;						
8	0.6924 ±0.0182	0.6789 ±0.0187	0.4701 ±0.0040	0.4027 ±0.0164	0.0003161	0.0178
10', NANase;						
10	0.6855 ±0.0148	0.6327 ±0.0153	0.4337 ±0.0047	0.5053 ±0.0159	0.0002069	0.0144
15', NANase;						
9	0.7254 ±0.0347	0.4433 ±0.0299	0.3216 ±0.0153	0.5029 ±0.0355	0.0010682	0.0327

significant difference, $\phi_{Hanks} - \phi_{15'\ NANase}$: $t = -1.968$ (DF = 14), $5.0\% > p > 2.5\%$ 0.T.

EXPERIMENT 17: c.80% growth, 0.025mg/ml. NANase;

control, 10' pre-incubation inHanks;

7	0.6405 ±0.0159	0.3872 ±0.0126	0.2480 ±0.0052	0.4448 ±0.0090	0.0002167	0.0147
5', NANase;						
7	0.6429 ±0.0377	0.5994 ±0.0378	0.3854 ±0.0090	0.4231 ±0.0280	0.0013259	0.0364
10', NANase;						
9	0.7467 ±0.0386	0.4267 ±0.0319	0.3186 ±0.0173	0.4788 ±0.0399	0.0013125	0.0362
15', NANase;						
10	0.7466 ±0.0191	0.4059 ±0.0158	0.3030 ±0.0089	0.4643 ±0.0203	0.0003163	0.0178

TABLE CONTINUED/

TABLE 5 Continued

EXPERIMENT 18: c. 70% growth, 0.025mg/ml. NANase;
control, 8' pre-incubation in Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
9	0.6800 ±0.0279	0.4159 ±0.0205	0.2828 ±0.0077	0.2996 ±0.0219	0.0006706	0.0259
3', NANase;						
8	0.6312 ±0.0168	0.6384 ±0.0174	0.4029 ±0.0024	0.2776 ±0.0127	0.0002670	0.0163
9', NANase;						
8	0.6344 ±0.0139	0.4397 ±0.0109	0.2789 ±0.0033	0.2914 ±0.0094	0.0001681	0.0130
13', NANase;						
8	0.6476 ±0.0119	0.5636 ±0.0112	0.3650 ±0.0027	0.3507 ±0.0092	0.0001306	0.0114

significant difference, $\phi_{\text{Hanks}} - \phi_{13' \text{ NANase}}$: $t = -2.1292$ (DF = 11), $5.0\% > p > 2.5\%$ one tail

in one experiment (exp.19).

a) Methods

(i) Measurement of growth density

Cells were plated onto seventeen 75cm^2 plastic bottles at five different densities. (Five bottles were plated at the lowest concentration and three bottles each were plated with four larger concentrations).

The cultures were grown for two days and then one bottles of each density was withdrawn and harvested to determine the cell density. Trypsin-EDTA was applied for 15 minutes to assure a yield of all cells growing in the bottles. The results are shown in table 6. All of the cultures were less than fully confluent.

(ii) Aggregation

The remaining 12 bottles were each washed four times in warm Hanks and then incubated at 37^{o}C in either Hanks or 0.025 mg/ml NANase in Hanks. After incubation, the medium was poured away and each culture was gently washed three times in tris at room temperature. The cells were harvested as usual and after two washes each with tris and then Hanks at 0^{o}C, the cells were resuspended in Hanks.

b) Results

The/

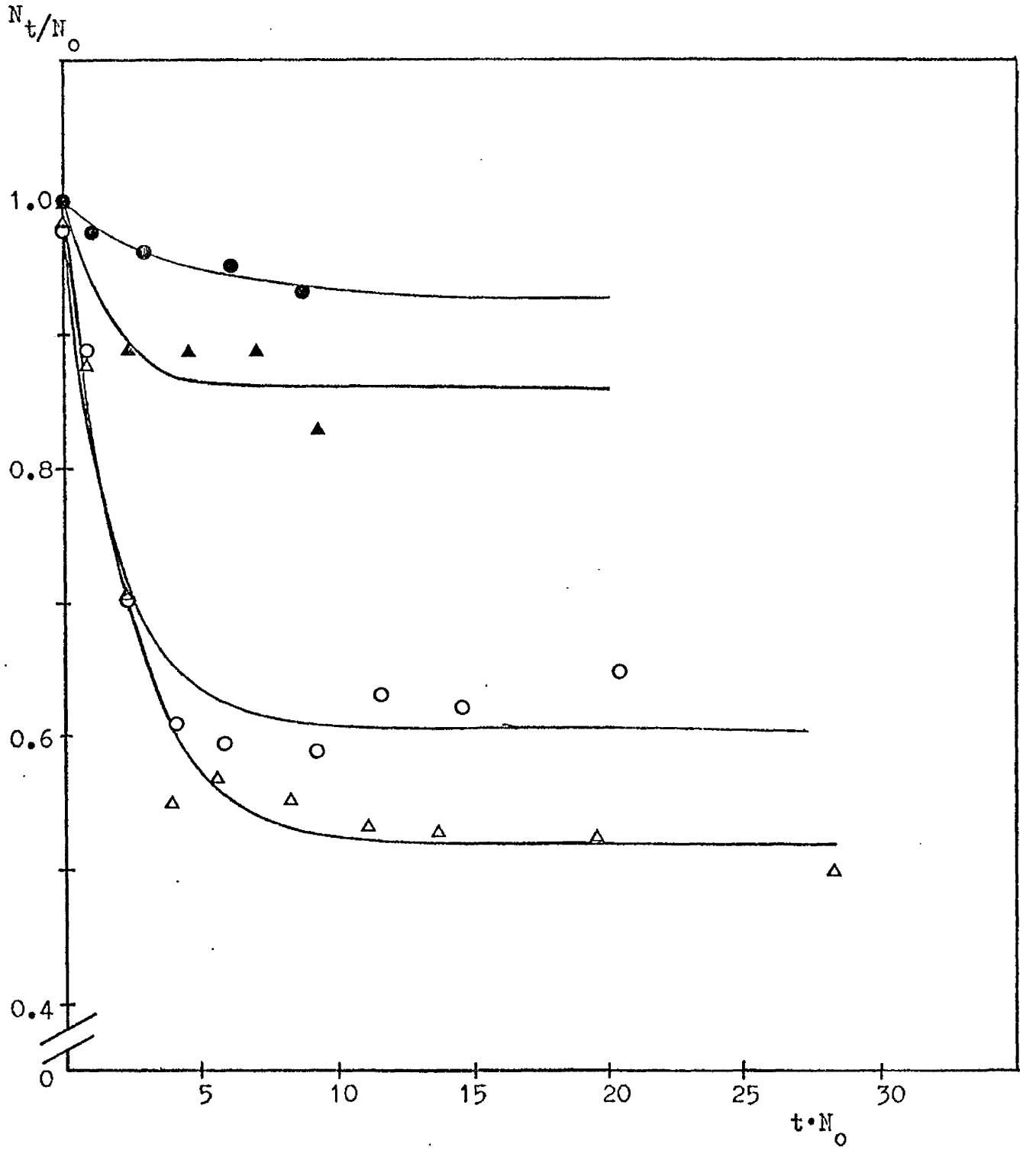


Figure 30. THE EFFECT OF NANASE ON THE AGGREGATION OF C-13 CELLS GROWN TO DIFFERENT DENSITIES, exp. 19:

NANase pre-treatment before aggregation,
growth density:

12.57×10^3 cells/cm^2-
- ● Hanks', control
- ▲ NANase treated

153.61×10^3 cells/cm^2-
- ○ Hanks', control
- △ NANase treated

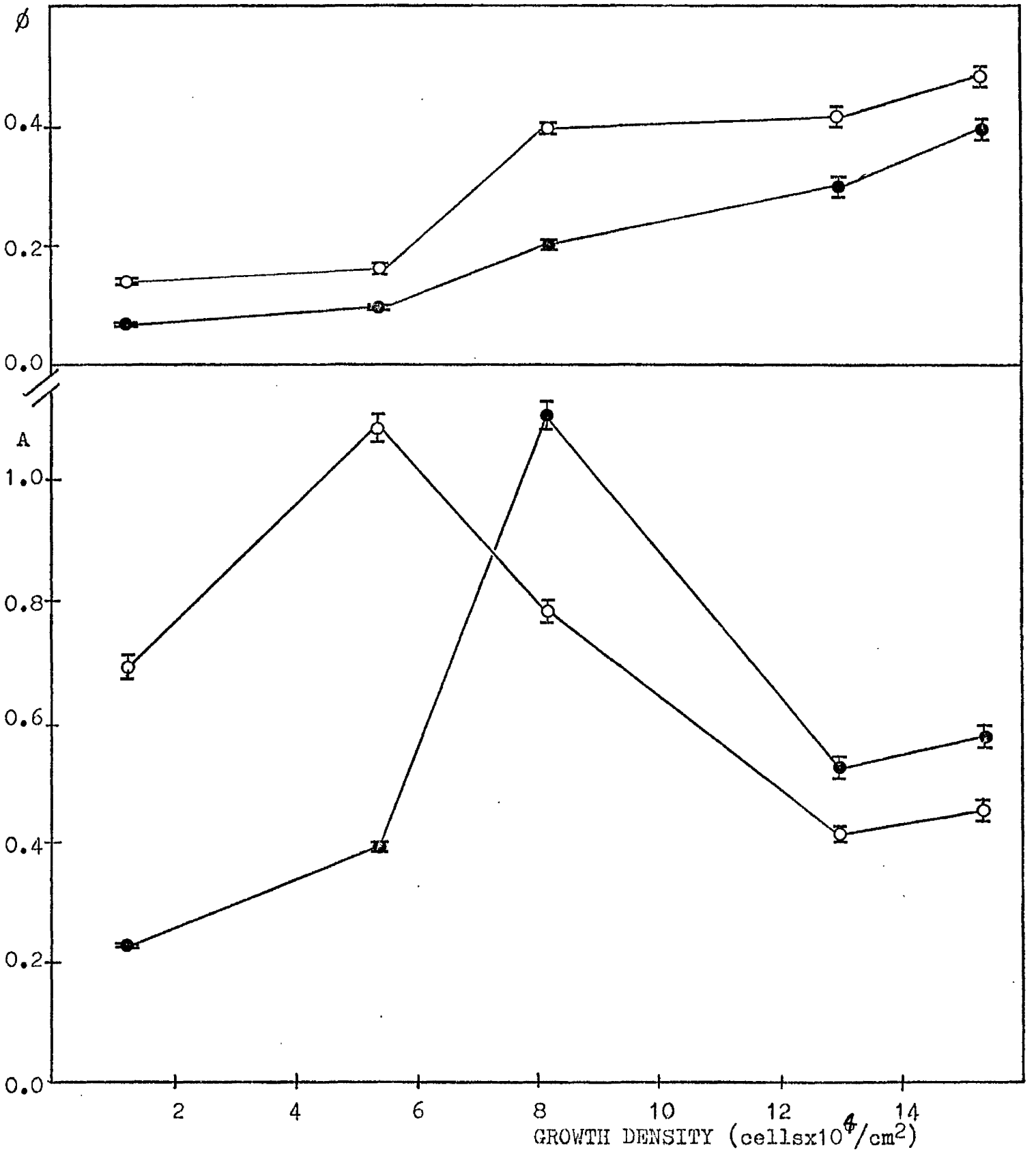


Figure 31a. THE EFFECT OF NANASE ON THE AGGREGATION OF C-13 CELLS GROWN TO DIFFERENT DENSITIES, exp. 19:

ordinate - the aggregation parameters,
bars indicate the standard errors of the points,
● Hanks treated ○ NANase treated.

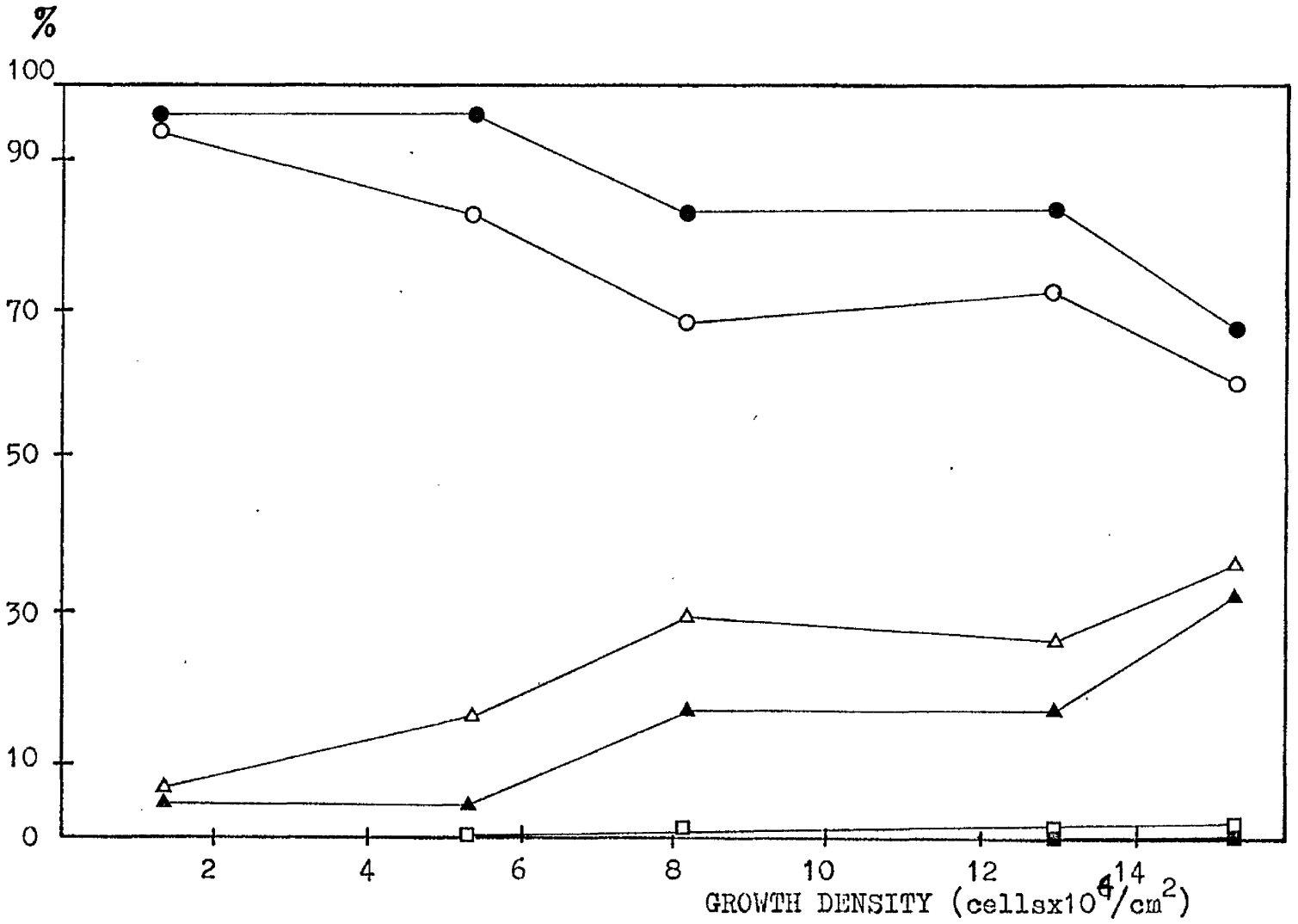


Figure 31b. THE EFFECT OF NANASE ON THE AGGREGATION OF C-13 CELLS GROWN TO DIFFERENT DENSITIES, exp. 19:

ordinate - the proportion of particles in the suspension made up of one size class, counted by hemocytometry,

size class:	single cells;	aggregates; ≤ 5 cells	aggregates. > 5 cells
Hanks treated:	●	▲	■
NANase treated:	○	△	□

TABLE 6

THE EFFECT OF GROWTH DENSITY AND NANASE PRE-TREATMENT ON C-13 AGGREGATION

Cultures were treated with 0.025mg/ml. NANase at 37°C for 10 minutes before aggregation. See text for details. The growth density of each control and experimental pair of cultures is given as thousands of cells/cm^2.

EXPERIMENT 19

n	No	A	α	ϕ	Residual Variance	SEp
Hanks/NANase. 12.573 cells x10^3/cm^2;						
5	0.5870 ±0.0047	0.2315 ±0.0019	0.1359 ±0.0002	0.0757 ±0.0011	0.0000187	0.0043
5	0.4636 ±0.0134	0.6950 ±0.0202	0.3222 ±0.0007	0.1398 ±0.0057	0.0001796	0.0134
53.867 cells x10^3/cm^2;						
5	0.4299 ±0.0095	0.3927 ±0.0088	0.1688 ±0.0006	0.0936 ±0.0035	0.0000891	0.0094
9	0.5246 ±0.0129	1.0941 ±0.0268	0.5740 ±0.0007	0.1677 ±0.0074	0.0001643	0.0128
81.667 cells x10^3/cm^2;						
8	0.5279 ±0.0121	1.0624 ±0.0244	0.5608 ±0.0008	0.1998 ±0.0071	0.0001460	0.0121
8	0.4950 ±0.0114	0.7859 ±0.0186	0.3890 ±0.0021	0.3929 ±0.0075	0.0001261	0.0112

TABLE CONTINUED/

TABLE 6 Continued

Hanks/NANase. 129.840 cells $x10^3/cm^2$;

n	No	A	α	ϕ	Residual Variance	SEp
8	0.6049 ±0.0158	0.5281 ±0.0147	0.3195 ±0.0031	0.2926 ±0.0111	0.0002233	0.0149
9	0.6857 ±0.0130	0.4077 ±0.0104	0.2795 ±0.0048	0.4064 ±0.0118	0.0001447	0.0120

153.613 cells $x10^3/cm^2$;

9	0.5803 ±0.0210	0.5894 ±0.0232	0.3420 ±0.0052	0.3902 ±0.0164	0.0004011	0.0200
10	0.5602 ±0.0169	0.4519 ±0.0183	0.2531 ±0.0069	0.4790 ±0.0139	0.0002386	0.0154

The aggregation curves for the lowest and highest densities used are shown in fig.30. Aggregation data for each density is given in table 6. NANase pre-treatment causes an increase in the values of $\emptyset$ between 20% and 100% of the control values as an inverse function of the growth density (see fig.31).

There is little if any effect of NANase pre-treatment on the rate constant A.

5) Discussion

a) The function of NANA in adhesion

There may be three possible explanations for the effect of NANase in promoting increased C-13 aggregation: (i) the enzyme binds to the cell surface and acts as a cell "cement"; (ii) the loss of NANA from the cell surface decreases the cell surface charge and thereby causes increased adhesion as predicted by the lyophobic colloid theory of cell adhesion (Curtis 1960); (iii) the loss of NANA reveals adhesion groups on the cell surface.

The first suggestion (i), is unlikely for two reasons, a) the purification of NANase normally involves specific adsorption and elution of the enzyme from the erythrocyte surface (Ada et al 1961). Therefore the enzyme is not known to bind irreversibly to cell surfaces at 37°C. b) Experiments

12/

12 - 18 show that the effect of NANase is a function of the time of incubation with the enzyme and rapid kinetics, expected for adsorption, are not followed.

The increase in aggregation following lowering of the cell surface charge is consistent with the lyophobic colloid theory of cell adhesion. However, this interpretation must be viewed in the light of the observation that EDTA had no effect on C-13 aggregation (fig.20).

Once NANA is removed from glycoproteins and glycolipids, then galactose, or N-acetyl-galactosamine, become the terminal residues of the sugar chains. These molecules may function as complementary adhesive groups. Roseman (1970) has suggested that, in neural retinal cells, terminal galactose functions to neutralize the adhesive properties of sugar chains, as NANA may function on C-13 cells.

b) Growth density

The effect of growth density on NANase promoted aggregation cannot be completely explained. From the limited amount of data it is not possible to draw entirely firm conclusions about the magnitude of the effect of density. It appears that NANase may increase the $\emptyset$ values by a constant amount at each growth density examined.

Oppenheimer et al (1969), Meezan et al (1969), and Hakamori/

Hakamori (1970) have suggested that the sugar composition of the cell surface is altered by cell surface contact (i.e. "contact modification", Hakamori (1970)). Therefore low growth density cells could possibly have a greater proportion of NANA-containing cell surface molecules, and NANase treatment should increase cell aggregation to a proportionally greater extent. Conversely high growth density cells will participate in more intercellular contact and have fewer NANA-containing groups on the cell surface. Thus NANase should affect their aggregation to a lesser degree. On the other hand, the faster growing cells (at low density) may have different amounts or types of cell surface components compared to cells in a slower growing dense population (see Warren 1969 and Conclusions).

c) NANase at t = o and on aggregates

The treatment of C-13 suspensions at t = o and pre-formed aggregates with NANase caused more aggregation than did pre-treatment of cultures. The reason for this may lie in the different methods used.

The cells or aggregates treated in suspension were first dispersed with trypsin-EDTA and then treated with NANase whereas the cultures were pre-treated with NANase before being suspended. Any effect on the cell surface charge or in the number of adhesive sites caused by NANase treatment of cells /

cells in culture might be reduced by the subsequent trypsin-EDTA procedure which would remove some of the possible new adhesive sites on glycoproteins revealing untouched NANA groups beneath; or, alternatively, reveal new charged groups beneath the trypsin sensitive protein. The results of NANase treatment of cells in aggregated suspensions may therefore be a more direct assay of the effect of the enzyme.

d) Other observations

Kemp (1968,1970) has claimed that NANase decreased the aggregation of chick embryonic cells. In his experiments extensively trypsinized cells were aggregated in a growth medium in the presence of NANase for several hours. However, Kemp (1970) found that pre-incubation of the cells in NANase had no effect on subsequent aggregation in comparison with the control. Therefore, the aggregation of chick embryonic cells may be caused by a different mechanism from that causing C-13 adhesion. The decreased aggregation caused by NANase may have been due to harmful physiological effects of the enzyme preparation apparent after long periods of exposure.

Weiss (1963) investigated the effect of NANase on cell disaggregation from glass by treating calf and rat cell lines with the enzyme before exposing the cell cultures to hydrodynamic shear. The enzyme treatment promoted greater cell dispersal but the results are difficult to compare with my own since /

since Weiss (1963) measured cell to glass adhesion.

6) Mechanism of NANase promoted aggregation

a) Aggregation at 0°C

(i) Method

In a series of seven experiments (exps. 20-26) cells were pre-treated with NANase in culture as before and then aggregated in Hanks at 0°C. Edwards and Campbell (1971a) found that C-13 aggregation was strongly temperature dependent. If NANase promotes increased cell aggregation by a different, perhaps temperature-insensitive mechanism, then pre-treated cells might aggregate to a greater extent at 0°C than the controls. The possible increase in aggregation could be as large as the absolute difference in $\emptyset$ between enzyme treated and untreated cells aggregated at 37°C.

(ii) Results

The aggregation curves for one experiment are shown in fig.32. It can be seen that there is no significant increase in aggregation at 0°C caused by NANase. The parameter data for each experiment is included in Table 7 together with the results of aggregation at 37°C for comparison. The initial, small and very rapid loss of count was usually observed in each experiment and may be due to a temperature-insensitive adhesive/

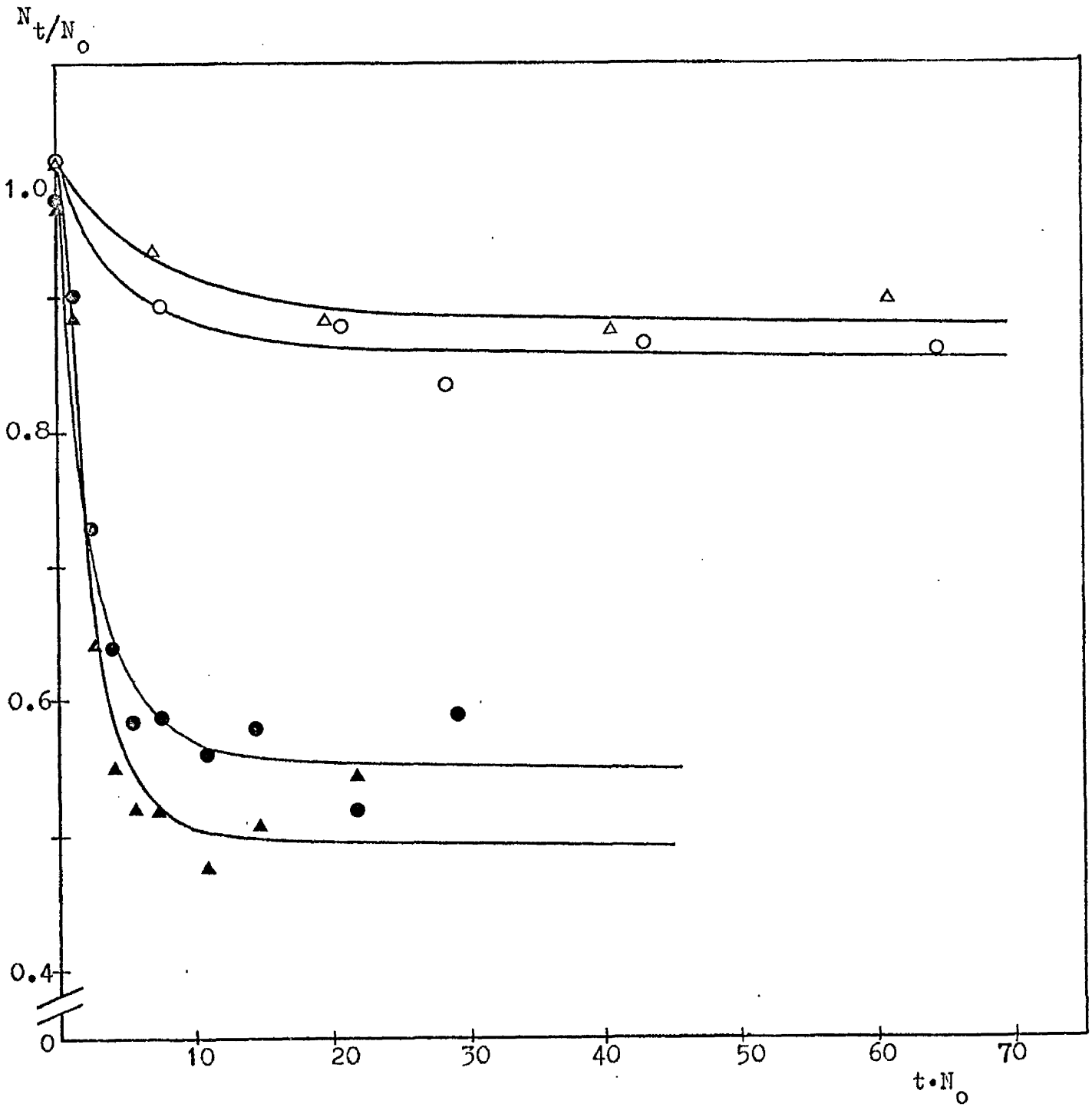


Figure 32. AGGREGATION OF NANASE PRE-TREATED C-13 CELLS AT 0°C, exp.25:

		aggregation temperature		NANase pre-incubation at 37°C before aggregation
exp. 25	○ –	0°C	–	Hanks control
	△ –	"	–	0.025mg/ml/15',
exp. 16	● –	37°C	–	Hanks control
	▲ –	"	–	0.025mg/ml/15',

TABLE 7

THE EFFECT OF PRE-TREATMENT WITH NANASE ON AGGREGATION AT 0°C.

The concentration of NANase and time of pre-incubation are given. See text for details. Immediately beneath the data for aggregation at 0°C is included for comparison the data for identical aliquots of the suspensions aggregated at 37°C.

n	No	A	α	ϕ	Residual Variance	SEp
EXPERIMENT 20: Hanks/NANase-0.025mg/ml, 30'; 0°C /;						
5	0.9355 ±0.0097	0.0311 ±0.0066	0.0291 ±0.0062	0.1071 ±0.0051	0.0000725	0.0085
*6	1.0380 ±0.0011	-	-	0.1170 ±0.0324	0.0010500	0.0324
37°C /, (exp. 6) Hanks/NANase;						
8	0.9663 ±0.0295	0.1315 ±0.0213	0.1271 ±0.0202	0.2704 ±0.0264	0.0006498	0.0255
8	1.0174 ±0.0334	0.1331 ±0.0296	0.1354 ±0.0297	0.3511 ±0.0335	0.0008445	0.0291
EXPERIMENT 21: Hanks/NANase-0.025mg/ml, 25'; 0°C /;						
*7	1.0570 ±0.0005	-	-	0.0655 ±0.0095	0.0000900	0.0095
*6	1.0020 ±0.0002	-	-	0.0641 ±0.0100	0.0001000	0.0100
37°C /, (exp. 7) Hanks/NANase;						
8	1.0458 ±0.0372	0.1832 ±0.0146	0.1916 ±0.0137	0.1927 ±0.0388	0.0011647	0.0341
8	0.9940 ±0.0195	0.1360 ±0.0146	0.1352 ±0.0142	0.3125 ±0.0181	0.0002924	0.0171

* Limited aggregation treatment

TABLE CONTINUED/

TABLE 7 Continued

EXPERIMENT 22: Hanks/NANase-0.05mg/ml. 30';

0°C /;

n	No	A	α	ϕ	Residual Variance	SEp
9	0.7980 ±0.0159	0.8861 ±0.0176	0.7071 ±0.0002	0.0945 ±0.0129	0.0002520	0.0159
7	0.8540 ±0.0248	1.2000 ±0.0349	1.0248 ±0.0001	0.1967 ±0.0231	0.0006165	0.0248
37°C /, (exp. 8) Hanks/NANase;						
12	0.9114 ±0.0223	0.2225 ±0.0209	0.2028 ±0.0184	0.4379 ±0.0300	0.0004185	0.0205
11	0.8169 ±0.0253	0.2986 ±0.0213	0.2439 ±0.0157	0.4583 ±0.0310	0.0005666	0.0238

EXPERIMENT 23: Hanks/NANase-0.05mg/ml. 30';

0°C /;

7	1.1137 ±0.0235	0.2032 ±0.0074	0.2264 ±0.0068	0.1769 ±0.0278	0.0005493	0.0234
7	1.0818 ±0.0196	0.1579 ±0.0051	0.1709 ±0.0046	0.1101 ±0.0212	0.0003763	0.0194
37°C /, (exp. 9) Hanks/NANase;						
11	1.1128 ±0.0148	0.2149 ±0.0141	0.2392 ±0.0153	0.5503 ±0.0272	0.0001922	0.0139
10	1.0802 ±0.0237	0.2106 ±0.0255	0.2275 ±0.0271	0.6133 ±0.0423	0.0004923	0.0222

TABLE CONTINUED/

TABLE 7 Continued

EXPERIMENT 24: Hanks/NANase-0.025mg/ml. 8';

0°C /;

n	No	A	α	ϕ	Residual variance	SEp
7	0.6530 ±0.0307	0.6710 ±0.0316	0.4381 ±0.0011	0.1024 ±0.0200	0.0009451	0.0307
7	0.7709 ±0.0347	0.3983 ±0.0185	0.3071 ±0.0037	0.1391 ±0.0275	0.0012031	0.0347
37°C /, (exp. 13) Hanks/NANase;						
10	0.8047 ±0.0248	0.2668 ±0.0245	0.2147 ±0.0186	0.5421 ±0.0259	0.0004882	0.0221
10	0.7235 ±0.0363	0.3068 ±0.0388	0.2220 ±0.0257	0.5868 ±0.0355	0.0010524	0.0324

EXPERIMENT 25: Hanks/NANase-0.025mg/ml. 15';

0°C /;

6	0.7157 ±0.0116	0.1858 ±0.0048	0.1330 ±0.0027	0.1408 ±0.0083	0.0001335	0.0116
5	0.6769 ±0.0100	0.1337 ±0.0045	0.0905 ±0.0028	0.1169 ±0.0062	0.0000982	0.0099
37°C / (exp. 16) Hanks/NANase;						
10	0.7254 ±0.0257	0.3661 ±0.0218	0.2656 ±0.0127	0.4458 ±0.0256	0.0005572	0.0236
9	0.7254 ±0.0347	0.4433 ±0.0299	0.3216 ±0.0153	0.5029 ±0.0355	0.0010682	0.0327

adhesive mechanism in a minority of the population or, more likely, aggregation of cell debris in the suspension. No aggregates were observed by hemocytometry and it is probable that this small loss of count is also a significant component of aggregation at 37°C.

b) NANase and trypsin

In two experiments the effects of NANase and trypsin on normally and heavily trypsin treated cells was investigated (exps. 27,28).

(i) Methods

In both experiments the control suspensions were harvested as usual. In exp.27, one culture bottle was treated with 0.05% Difco trypsin-EDTA for 10 minutes whereas in exp.28 one bottle was harvested with 0.5% trypsin-EDTA for 10 minutes. The cells were washed twice each in tris and Hanks and re-suspended in Hanks or NANase at 0°C. Aggregation was carried out at 37°C.

After aggregation halted, 0.1 ml. of Difco trypsin (final concentration 0.025%) was added to each flask.

(ii) Results

As seen previously in exp.10, NANase, when added to normally harvested cells at t = o, increased $\emptyset$ by at least 50% (see discussion previously on effect of NANase at t = o and on aggregates). The rate constant was somewhat decreased in /

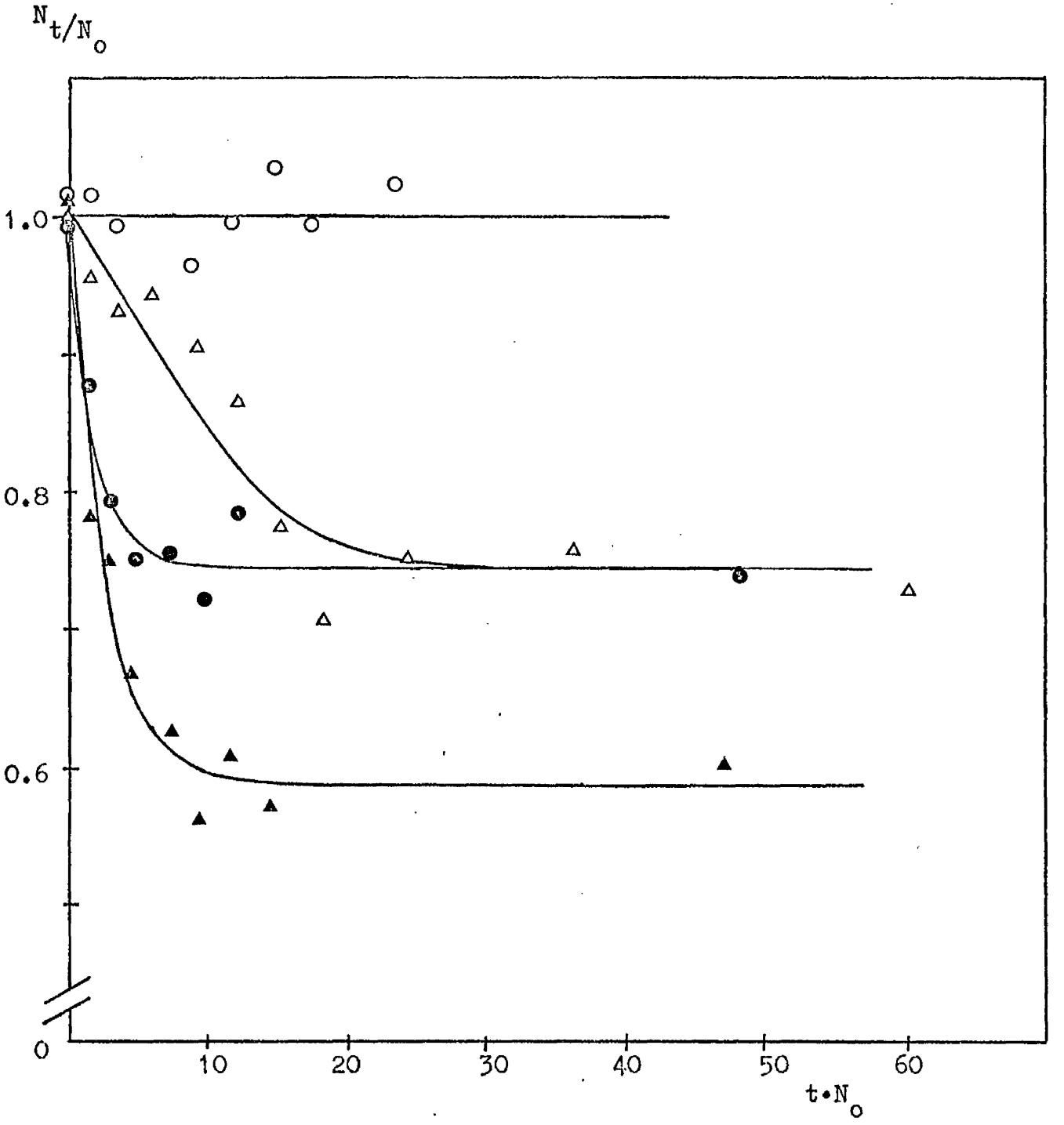


Figure 33. THE EFFECT OF NANASE ON NORMALLY AND HEAVILY TREATED C-13 CELLS, exp. 28:

	Hanks control	NANase added (0.032mg/ml.) at t = 0,
normal trypsin:	●	▲
heavy trypsin:	○	△

TABLE 8

THE EFFECT OF NANASE ON TRYPSIN PRE-TREATED C-13 CELL SUSPENSIONS

The normally or heavily trypsinized cell suspensions were resuspended ineither Hanks or the indicated concentration of NANase at t = 0. See text for details.

EXPERIMENT 27: 0.025mg/ml. NANase;

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
11	0.7585 ±0.0223	0.5423 ±0.0185	0.4114 ±0.0070	0.4125 ±0.0244	0.0004826	0.0220

control, NANase;

12	0.7716 ±0.0251	0.3854 ±0.0265	0.2973 ±0.0180	0.6306 ±0.0358	0.0005776	0.0240

heavily trypsinized, Hanks;

10	0.7521 ±0.0285	0.5095 ±0.0221	0.3832 ±0.0080	0.3441 ±0.0281	0.0007827	0.0280

heavily trypsinized, NANase;

10	0.7148 ±0.0297	0.2159 ±0.0426	0.1543 ±0.0298	0.5777 ±0.0249	0.0006760	0.0260

EXPERIMENT 28: 0.032mg/ml. NANase;

control, Hanks;

8	0.4831 ±0.0118	0.5376 ±0.0139	0.2597 ±0.0022	0.2548 ±0.0065	0.0001350	0.0116

control, NANase;

9	0.4708 ±0.0146	0.3950 ±0.0177	0.1860 ±0.0060	0.4116 ±0.0092	0.0001966	0.0140

heavily trypsinized, Hanks (two suspensions);

8	0.6047 ±0.0097	0.7246 ±0.0116	0.4382 ±0.0004	0.0898 ±0.0058	0.0000931	0.0096
*8	0.5805 ±0.0059	-	-	0.0000 ±0.0059	0.0002820	0.0168

*No detectable aggregation, See p109 for treatment

in exp.27 and 28, compared with A in the controls. This is probably the result of the effect of NANase on the adhesiveness of the cells; the value of A in NANase treated suspensions is both a function of the collision efficiency of the cells as it is modified in time by the enzyme and depends on ϕ .

The heavily trypsinized control in exp.28 displays no aggregation. However, addition of NANase causes aggregation at a relatively low rate, which proceeds until ϕ approaches that of the normally harvested control (fig.33).

Essentially, the same results were obtained with exp.27, but in this experiment the "heavy" trypsinization was insufficient to produce a completely non-adhesive suspension. Therefore, the results are not clearly interpretable (Table 8). It was found that all the aggregates in each suspension were dispersed by the addition of trypsin once aggregation had ceased.

7) Discussion

The results of experiments 6-28 indicate that the mechanism of enhanced adhesion caused by NANase cannot be differentiated from that which causes adhesion in untreated cell by the criteria of temperature and trypsin sensitivity of aggregation.

The additional aggregation of extensively trypsinized cells caused by NANase might indicate that the NANA was removed from /

from newly accessible, trypsin insensitive (e.g. glycolipid) sites. The observation that these adhesions were also sensitive to trypsin conflicts with this conclusion; but the results could be explained if a second, trypsin sensitive, adhesive component existed on the cell surface. Roseman (1970) has suggested that the adhesion of chick embryo neural retinal cells requires two types of cell surface components.

EXPERIMENTAL IV : THE EFFECTS OF L-GLUTAMINE AND COMPLEX MEDIA ON AGGREGATION.

A. Aggregation in Medium 199

1) Methods

In an experiment, similar to II 1 above, aliquots of a cell suspension were aggregated in several, individual flasks and then a small amount of trypsin was added to each flask. As before, disaggregation was interrupted after two minutes by the addition of ti. The experiment differed from II 1 in that the medium consisted of Hanks supplemented with 50% Medium 199[38].

2) Results

After the addition of ti a low rate of aggregation could be detected in all flasks (fig.34) unlike the behaviour of cells in Hanks alone. The regression rates, correlation coefficient and their significance are listed in Table 9. All the rates are similar with strong and significant correlation. Hemocytometry confirmed that the decrease in Ø was due to the formation of aggregates.

B. Generation of adhesiveness

The aggregation promoting effect of the complex medium was further investigated in two ways. Both normally harvested cells and those made non-adhesive by pre-treatment with high levels of trypsin were examined. First, cells were harvested with /

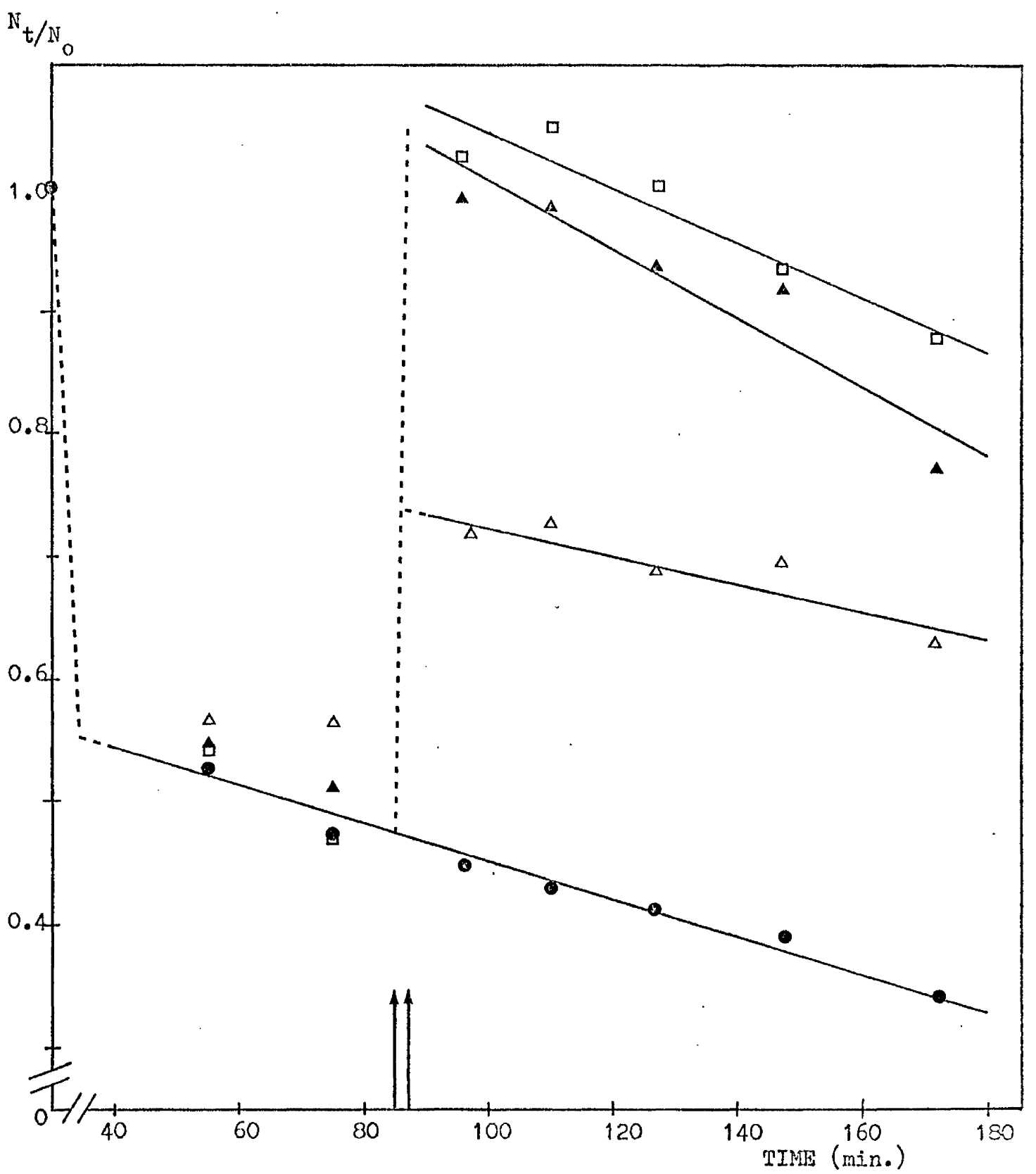


Figure 34. RE-AGGREGATION OF TRYPSIN TREATED C-13 CELLS IN MEDIUM 199, exp. 1:

The suspension are aggregated in a medium composed of Hanks 50%/ Medium 199 50%. Trypsin added at the first arrow, ti added at the second arrow. Best fit regression lines.

flask: trypsin (for 2'):

- ● - Hanks control
- △ - 450 BAEE units of trypsin/ml.
- ▲ - 1800 " " " " "
- □ - 9000 " " " " "

TABLE 9

AGGREGATION OF TRYPSINIZED CELLS IN MEDIUM 199

The amount of trypsin added to the cell suspension after aggregation is given. The treatment is for linear regression, see page 110. The number of points, n; rate of regression, c; correlation coefficient, r; and its significance are included. See text for details.

EXPERIMENT 1

	n (after ti)	c (particles) ($\times 10^6$/ml/min)	r	p
control :	7	-0.0011	-0.976	0.05% >p
900 BAEE units/ml.:	5	-0.00280	-0.944	0.5%>p>0.1%
450 BAEE units/ml.:	5	-0.00114	-0.908	2.5%>p>1.0%
9000 BAEE units/ml. :	5	-0.00222	-0.953	1.0%>p>0.5%

with trypsin and pre-incubated for various times in Eagles medium. Aggregation was tested after the cells had been washed free of the medium and resuspended in Hanks. In this way, the effect of Eagles incubation on the parameters A and $\emptyset$ could be measured. Secondly, the components of Eagles medium were tested for their aggregation promoting activity.

1) Pre-incubation of C-13 cells in Eagles medium

a) Methods

In the first of three experiments (exps.2-4) one bottle of C-13 cells was dispersed as usual while another was harvested with a 10-fold greater concentration of Difco trypsin (0.5%) for 5 minutes. Both suspensions were washed three times in tris and then resuspended in a small volume of Hanks with 100 μg/ml. ti. The suspensions were then added to 1 liter flasks containing 600 mls. of Eagles medium at 37°C, gassed continually with 5% CO_2 in air, and stirred gently with a magnetic stirring bar. All procedures, glassware, media and gas were sterile.

The suspensions were composed almost totally of single cells at a concentration of 4×10^3/ml. This low concentration was necessary to reduce the collision frequency during incubation.

In two other experiments virtually the same procedure was followed except that in exp.3 1% calf serum was added to the Eagles and pure trypsin was used (activity 600 and 6000 BAEE units/

units/ml. respectively). In exp.4, 5% calf serum and 5% TPB were added to the Eagles. The more heavily trypsinized suspension was exposed to 7200 BAEE units/ml. of pure trypsin.

At intervals after the start of incubation 100 ml. aliquots of each suspension were withdrawn and immediately placed in two 50ml. conical centrifuge tubes on ice. The cells were washed twice in Hanks at 0°C and then re-suspended in Hanks in 10 ml. shaker flasks. Aggregation was at 37°C.

b) Results

The increase in aggregation caused by pre-incubation in Eagles medium is shown for one experiment in fig.35. Data for each experiment are included in Table 10. In each experiment, the aggregation of the control suspension increased slightly but the values of $\emptyset$ and A for the heavily trypsinized suspension increased at a greater rate and approached the values of the control suspensions (figs. 36,37). Hemocytometry showed that the decrease in N_t for all suspensions was due to aggregation.

2) Discussion

The results indicate that heavily trypsinised cells regain adhesiveness when incubated in a complex medium. The origin of newly adhesive surface may be: 1) by the adsorption of medium components onto the cell surface, or 2) through metabolic synthesis and incorporation of adhesive components into the cell surface.

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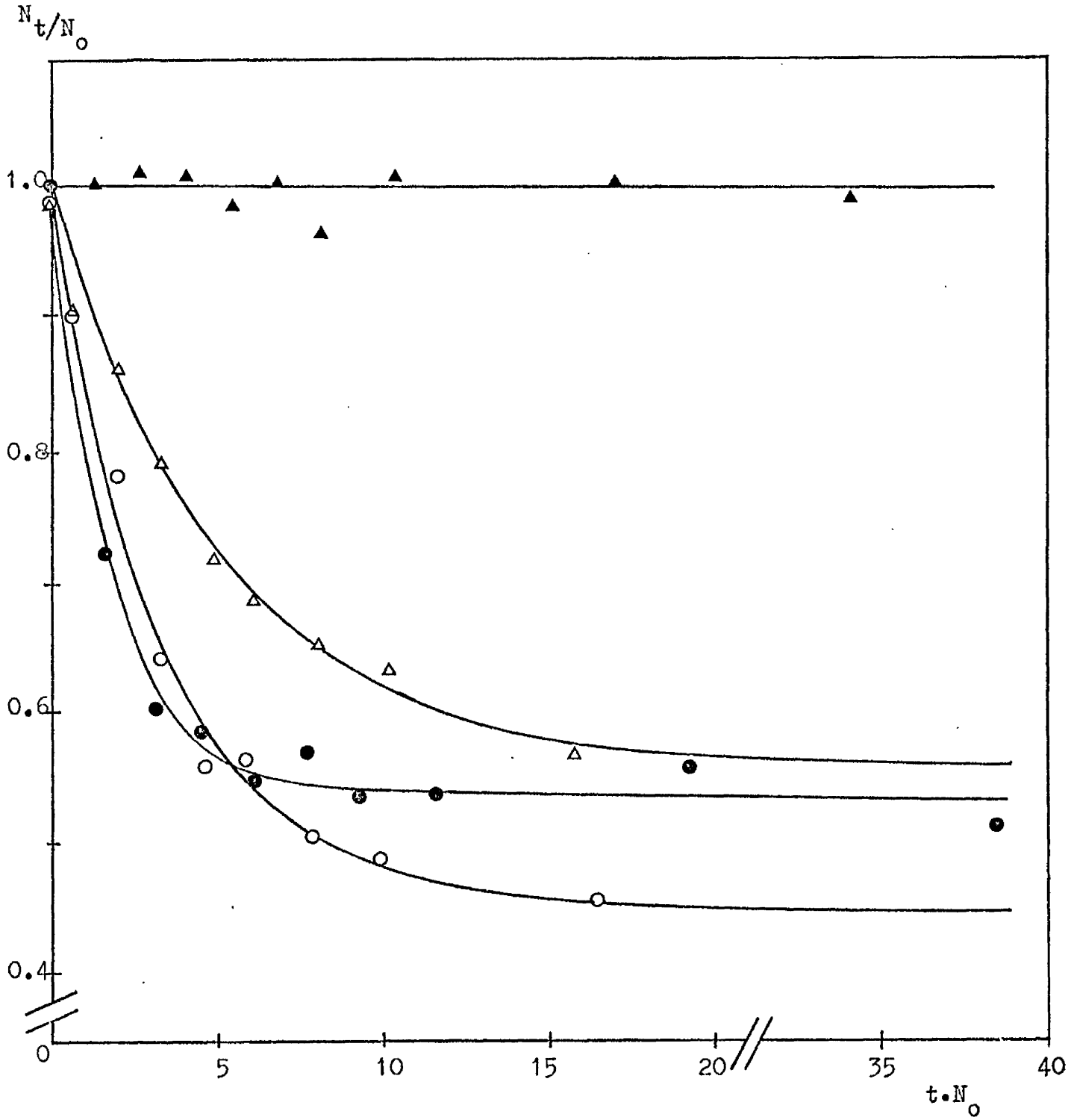


Figure 35. AGGREGATION OF NORMALLY AND HEAVILY TRYPSIN TREATED C-13 CELLS PRE-INCUBATED IN EAGLES MEDIUM, exp. 4:

The cells were either dispersed as usual or with enough trypsin to inhibit subsequent aggregation.
Pre-incubation in Eagles Medium at 37°C before aggregation in Hanks.

Time of pre-incubation:	0'	180'
normal trypsin:	●	○
heavy trypsin:	▲	△

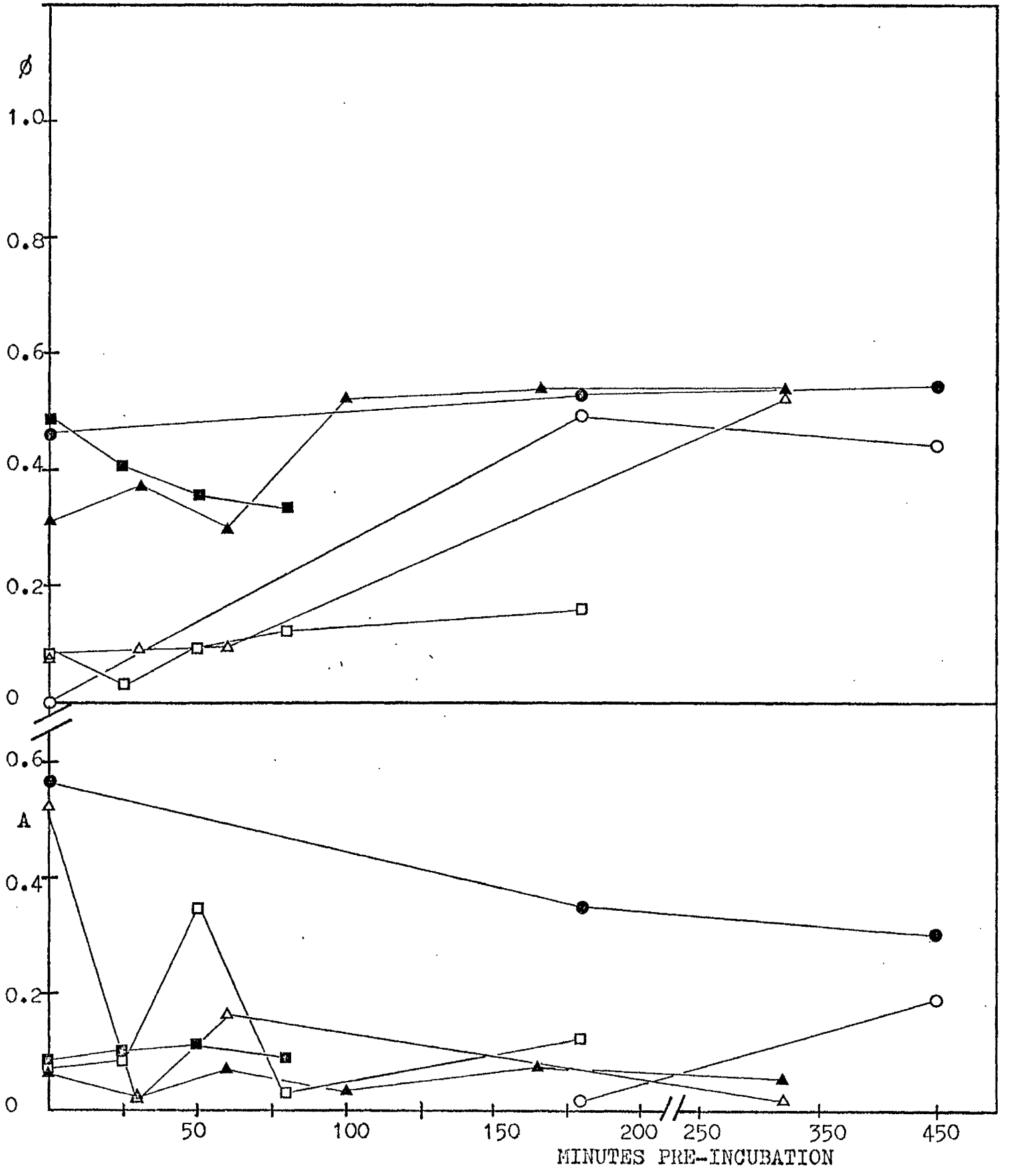


Figure 36,37. AGGREGATION OF NORMALLY AND HEAVILY TRYPSIN TREATED C-13 CELLS PRE-INCUBATED IN COMPLEX MEDIA:
ordinate - the parameters of aggregation,
experiment: 2 3 4
normal trypsin: ■ ▲ ●
heavy trypsin: □ △ ○

TABLE 10

PRE-INCUBATION OF C-13 CELLS IN EAGLES MEDIUM

The length of time of pre-incubation for the control and heavily trypsinized suspensions is given. See text for complete details.

EXPERIMENT 2: standard tryosin/ heavy trypsin;

t = 0;

n	No	A	α	ϕ	Residual Variance	SEp
10	1.2384 ±0.0249	0.0897 ±0.0272	0.1111 ±0.0336	0.3915 ±0.0278	0.0004350	0.0209
10	1.3882 ±0.0277	0.0714 ±0.0070	0.0990 ±0.0095	0.0868 ±0.0291	0.0005198	0.0228
t = 25';						
9	1.1229 ±0.0216	0.1057 ±0.0235	0.1187 ±0.0263	0.4009 ±0.0241	0.0003437	0.0185
9	1.1714 ±0.0269	0.0856 ±0.0032	0.1003 ±0.0030	0.0344 ±0.0249	0.0005149	0.0227
t = 50';						
9	1.1100 ±0.0147	0.1101 ±0.0142	0.1223 ±0.0157	0.3575 ±0.0162	0.0001602	0.0127
9	1.1464 ±0.0119	0.3484 ±0.0038	0.3994 ±0.0013	0.0921 ±0.0137	0.0001377	0.0117
t = 80';						
8	1.1502 ±0.0193	0.0933 ±0.0192	0.1074 ±0.0220	0.3388 ±0.0170	0.0002566	0.0160
8	1.1304 ±0.0404	0.0296 ±0.0158	0.0335 ±0.0178	0.1266 ±0.0100	0.0008771	0.0296
t = 180', heavily trypsinized suspension;						
9	1.1684 ±0.0222	0.1282 ±0.0090	0.1498 ±0.0102	0.1627 ±0.0242	0.0003821	0.0195

TABLE CONTINUED/

TABLE 10 Continued

EXPERIMENT 3: standard trypsin/ heavy trypsin;

t = 0;

n	No	A	α	ϕ	Residual Variance	SEp
10	0.8436 ±0.0474	0.0630 ±0.0507	0.0531 ±0.0427	0.3110 ±0.0186	0.0012150	0.0349
8	0.8759 ±0.0255	0.5210 ±0.0152	0.4564 ±0.0014	0.0854 ±0.0223	0.0006391	0.0253

t = 30';

9	0.9654 ±0.0382	0.0270 ±0.0395	0.0261 ±0.0381	0.3777 ±0.0045	0.0006664	0.0258
8	0.9535 ±0.0076	0.0210 ±0.0020	0.0200 ±0.0019	0.0931 ±0.0006	0.0000457	0.0068

t = 60';

8	0.9443 ±0.0558	0.0735 ±0.0693	0.0694 ±0.0653	0.3063 ±0.0441	0.0022426	0.0474
6	0.8674 ±0.0023	0.1665 ±0.0011	0.1444 ±0.0008	0.0974 ±0.0024	0.0000381	0.0062

t = 100';

7	0.9301 ±0.0395	0.0348 ±0.0623	0.0323 ±0.0579	0.5212 ±0.0070	0.0009490	0.0308

*6 0.9887 - , $c = -0.0017 \times 10^6$ particles/min., $r = -0.8566$ ($0.025 > p > 0.01$)

t = 165';

7	0.8199 ±0.0359	0.0788 ±0.0727	0.0646 ±0.0595	0.5330 ±0.0196	0.0009010	0.0300

*6 0.9140 - , $c = -0.0025 \times 10^6$ particles/min., $r = -0.9958$ ($0.0005 > p$)

t = 320';

7	0.6996 ±0.0276	0.0543 ±0.0558	0.0380 ±0.0390	0.5336 ±0.0061	0.0004672	0.0216
7	0.9000 ±0.0158	0.0194 ±0.0280	0.0174 ±0.0252	0.5271 ±0.0014	0.0001315	0.0115

*Linear regression, p 110

TABLE CONTINUED/

TABLE 10 Continued

EXPERIMENT 4: standard trypsin/ heavy trypsin;

t = 0';

n	No	A	d	ϕ	Residual Variance	SEp
10	0.7679 ±0.0132	0.5656 ±0.0112	0.4343 ±0.0043	0.4589 ±0.0150	0.0001692	0.0130
*9	0.6818 ±0.0036	-	-	0.0000 ±0.0036	0.0001144	0.0107

t = 120';

9	0.7607 ±0.0241	0.3562 ±0.0194	0.2709 ±0.0120	0.5245 ±0.0226	0.0004396	0.0210
9	0.5631 ±0.0078	0.0195 ±0.0023	0.0110 ±0.0013	0.4965 ±0.0000	0.0000279	0.0053

t = 450';

9	0.6583 ±0.0161	0.3033 ±0.0155	0.1996 ±0.0089	0.5436 ±0.0107	0.0001795	0.0134
9	0.6735 ±0.0110	0.1952 ±0.0097	0.1315 ±0.0062	0.4408 ±0.0050	0.0000741	0.0086

* Treatment for no detectable aggregation, see p 109

The first suggestion may be discounted for two reasons. First, the cells were washed free of the medium before being re-suspended in Hanks for aggregation. Secondly, an adsorption mechanism would presumably operate instantly but the effect of incubation was observed to continue to increase aggregation as a function of incubation time. This was also seen in exp.1.

Therefore the re-aquisition of adhesiveness by the cells is due to the appearance of new, adhesive components on the cell surface. Presumably this regeneration is through the normal process of cell surface synthesis and incorporation (Warren and Glick 1968, Pasternak and Friedrichs 1970, Kraemer 1967).

3) Active components of Eagles medium

a) Methods

In two preliminary experiments (exps.5,6) the components of Eagles medium were tested for their activity in increasing cell aggregation. Fresh, low passage cells were grown for two days in several 120cm^2 glass bottles and harvested at low density. The cultures were harvested as usual and re-suspended at approximately 5 x 10^5/ml. in three mls. of Hanks in each shaker flask. Aggregation was at 37^{o}C.

The Hanks of the first experiment was buffered with tris as usual but in the second experiment HEPES[39] was used (4670mg/ liter)/

liter) because of the possible sensitivity of some cellular enzymes to tris.

Tables 11 and 12 list the substances used in each experiment.

b) Results

Of the components of Eagles medium only L-glutamine consistently promoted aggregation, see fig.38. The other amino acids, although causing aggregation in one experiment, failed to do so in the second. The same was true of NANA. No conclusions can be drawn about the effect of these two substances until more experiments are completed.

4) Discussion

The function of L-glutamine in increasing cell adhesiveness may be to supply the amino groups necessary for the synthesis of amino sugars which are required as adhesive cell surface components. This conclusion is supported by the following evidence. 1) The metabolic inhibitor fluoride prevented the effect. 2) The specific analogue of L-glutamine, azaserine, inhibited the activity of the amino acid. (In exp.5 the ratio of L-glutamine to azaserine may have been too high). 3) Oppenheimer et al (1969, see previously) have examined the effects of the constituents of medium 199 on teratoma cell aggregation and have concluded that L-glutamine (and the amino sugar/

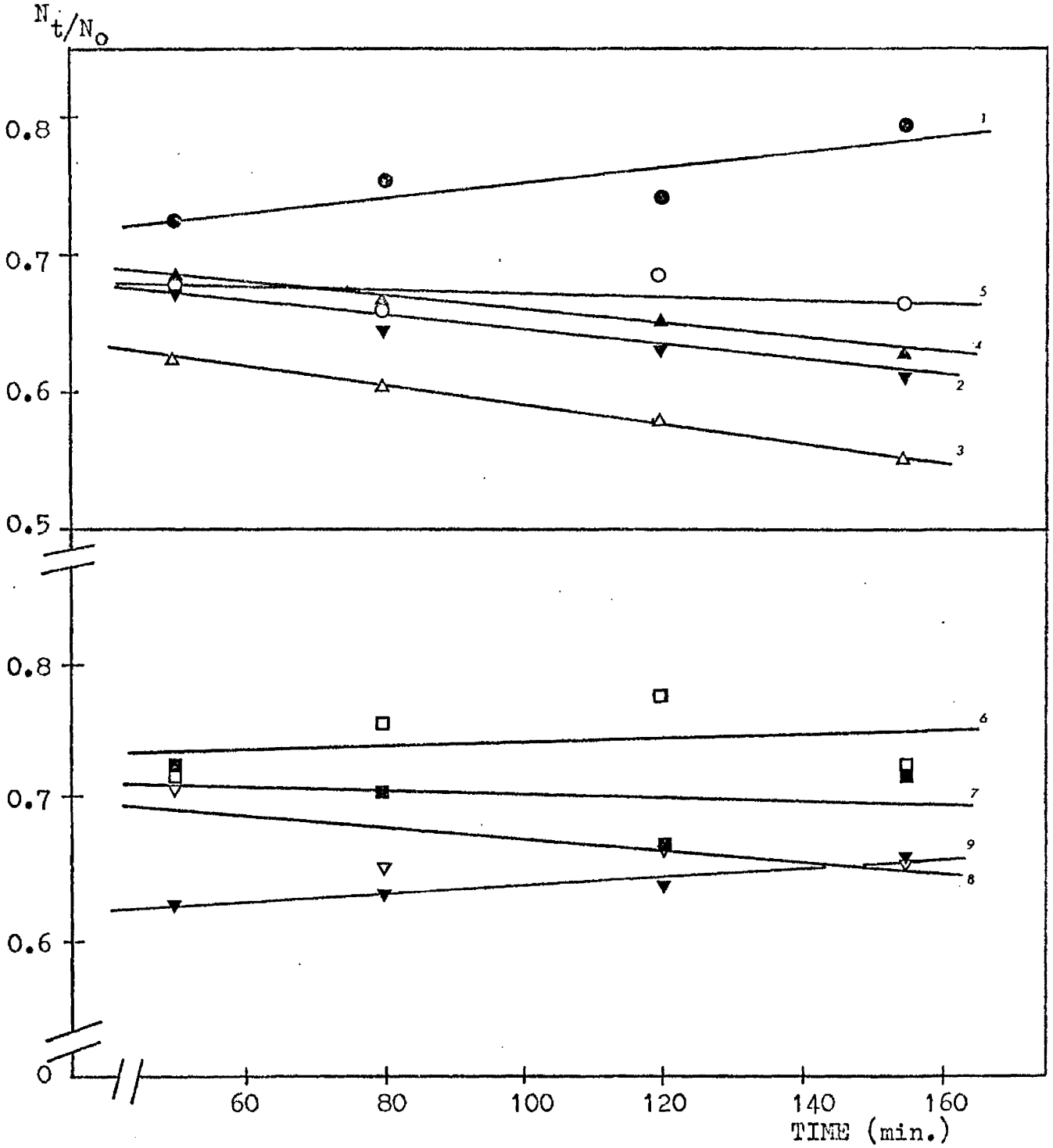


Figure 38. AGGREGATION PROMOTING ACTIVITY OF COMPONENTS OF EAGLES MEDIUM AND OTHER SUBSTANCES, exp. 6:

abscissa - time after initiation of aggregation, substances added at t = o',

1 ● Hanks' control
2 ▼ glutamine, 0.22mM
3 △ " , 1.10mM
4 ▲ " , 5.52mM
5 ○ Eagles amino acids 1.1x normal conc.
6 □ glucosamine, 7.13mM
7 ■ mannosamine, "
8 ▽ glutamine, 5.52mM + NaF, 1.99mM
9 ▼ glutamine, 5.52mM + azaserine, 14.38mM

TABLE 11

EXPERIMENT 5

AGGREGATION PROMOTING ACTIVITY OF THE COMPONENTS OF EAGLES MEDIUM AND OF SOME OTHER SUBSTANCES

The concentration of each component is given. The superscripts refer to Appendix I: Materials. Linear regression is applied to the points, n, after the addition of the substances at 15'. See p 110.

COMPONENT	CONCENTRATION (mM)	n	c (particles x10^6/ml/min)	r	p (one tail.)
1) Hanks', control:	-	7	+0.00001	+0.094	p >5.0%
2) Eagles vitamines[40]:	normal	6	-0.00002	-0.076	p >5.0%
3) Eagles amino acids[41]:	$\frac{2}{3}$normal	4	-0.00071	-0.942	5.0%>p>2.5%
4) Amino sugars: N-acetyl-glucosamine[42], " mannosamine[43], " galactosamine[45], galactosamine[44].	5.91, 6.00, 6.01, 5.90.	7	+0.00040	+0.954	0.05% >p
5) NANA[37]:	0.61	4	-0.00057	-0.996	0.25%>p>0.05%
6) glutamine[46]:	1.00	4	-0.00070	-0.925	5.0% >p > 2.5%
"	2.01	4	-0.00069	-0.986	1.0% >p>0.5%
"	3.80	4	-0.00091	-0.969	2.5% >p>1.0%
"	6.01	4	-0.00100	-0.823	p >5.0%
"	10.01	4	-0.00131	-0.993	5.0% >p >2.5%
7) glutamine, + azaserine[47]:	5.00 10.27	4	-0.00104	-0.973	2.5% >p>1.0%
8) glutamine, + azaserine, + mannosamine[56].	5.00 10.27 6.20	4	-0.00131	-0.983	1.0% >p>0.5%
9) glutamine, + azaserine, + glucosamine[55].	5.00 10.27 6.20	4	-0.00135	-0.951	2.5% >p>1.0%
10) glucosamine:	6.20	4	+	-	-
11) mannosamine:	6.20	4	+	-	-

TABLE 12

EXPERIMENT 6
AGGREGATION PROMOTING ACTIVITY OF THE COMPONENTS OF EAGLES MEDIUM AND OF SOME OTHER SUBSTANCES

The concentration of each substance is given, each is added at t = 0'. Linear regression is applied to the points after 50'. n = 4.

COMPONENT	CONCENTRATION (mM)	c (particles $\times 10^6$/ml/min)	r	p (one tail)
1) Hanks', control:	--	+0.00055	+0.845	p > 5.0%
2) Sugars:		-0.00007	-0.464	"
glutamic acid[48],	5.74			
galactose[49],	5.74			
lactose[50],	5.74			
mannose[51],	5.74			
L-fucose[52],	5.75			
D-fucose[52],	5.73			
3) glutamic acid, + NH_4^+[53],	5.74 : 5.75	-0.00002	-0.115	"
4) inositol[54]:	7.41	-0.00001	-0.019	"
5) mannosamine:	7.13	-0.00013	-0.220	"
6) NANA:	5.72	-0.00043	-0.816	"
7) glucosamine:	7.13	+0.00012	+0.201	"
8) glutamine:	0.22	-0.00058	-0.989	1.0% > p > 0.5%
9) " :	1.10	-0.00070	-0.996	0.25% > p > 0.05%
10) " :	5.52	-0.00052	-0.998	"
11) Eagles amino acids:	1.1xnormal	-0.00005	-0.210	p > 5.0%
12) N-acetyl-neuramin-lactose[57]:	5.72	-0.00024	-0.839	"
13) glutamine, + NaF[58],	5.52 : 1.99	-0.00043	-0.737	"
14) glutamine, + azaserine,	5.52 : 14.38	+0.00031	+0.960	2.5% > p > 1.0%

sugars D-glucosamine and D-mannosamine) functions to increase adhesion by donating amino groups to amino sugars which may be incorporated into cell surface molecules necessary for cell adhesion as discussed above.

The kinetics of the effect of L-glutamine on C-13 and teratoma cells seem similar. However, the failure of D-glucosemine and D-mannosamine to promote aggregation of C-13 cells is surprising (in fact D-glucosamine may have caused disaggregation). There is no reason at present to believe that the enzymes for their utilization in C-13 cells are either lacking or damaged by the harvesting and incubation procedures. D-glucosamine has been shown to be incorporated into cellular macromolecules by teratoma cells (Oppenheimer et al 1969), and by C-13 cells (Kraemer 1966, Allen and Snow 1970).

C. Comparison of the Effects of L-glutamine and Eagles Medium

1) Methods

In order to assess the efficiency of L-glutamine in comparison with Eagles medium in causing increased aggregation two experiments (exps. 7,8) were carried out using heavily trypsinized, non-adhesive cells.

In exp.7 the cells were harvested using a 1% Difco trypsin solution for 6 minutes. The cells were washed by centrifugation four times in 0°C tris and then re-suspended in either/

either Hanks, 50% Hanks - 50% Eagles, or 5mM/ml. L-glutamine in Hanks. In exp. 8 the cultures were harvested with 0.5% Difco trypsin for 4 minutes and washed as before. The cells were re-suspended according to Table 13. Re-aggregation was at 37°C.

2) Results

In both experiments there was a delay of from one to two hours before any aggregation occurred. It may be that the trypsin treatment was so extreme that many cellular processes were inhibited and it is the period of their repair which results in the observed aggregation lag. Similar lags have been found to occur in heavily trypsinized chick embryonic tissue (Roth and Weston 1967).

L-glutamine promoted aggregation at a constant, low rate as a function of the concentration of the amino acid (see fig. 39 and Table 13). However, Eagles containing medium which in fact had less L-glutamine (1.31mM for modified Eagles medium 50% + 50% Hanks, also supplemented with 1.0 mM L-glutamine in exp.2) resulted in greater aggregation. Since the amino acid fraction of modified Eagles medium (without L-glutamine)caused little if any aggregation (exps.5,6) the function of complete medium may be to generally enhance cellular physiology and the utilization of L-glutamine.

D./

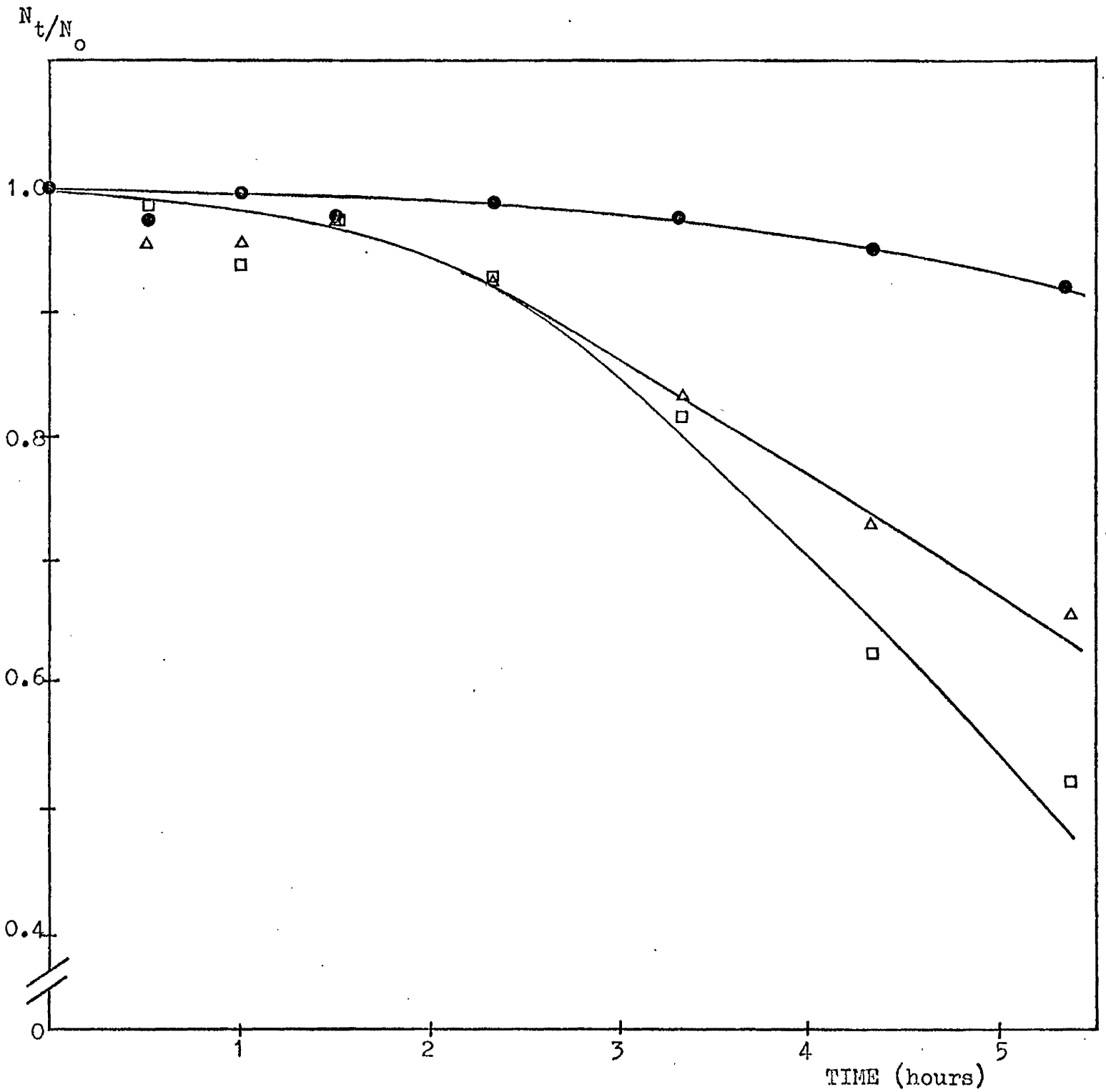


Figure 39. COMPARISON OF THE EFFECT OF L-GLUTAMINE AND EAGLES MEDIUM, exp. 7:
Aggregation was inhibited by heavy trypsin pre-treatment, the cells were suspended in the media indicated at t = 0', ● Hanks' control; △ 5mM glutamine; □ Eagles medium 50%, Hanks 50%

TABLE 13

COMPARISON OF THE EFFECTS OF L-GLUTAMINE AND EAGLES MEDIUM ON HEAVILY TRYPSINIZED CELLS

The heavily trypsinized cells are suspended in the medium indicated at t = 0. The treatment is for linear regression as before: the points are after the lag phase. See text for details.

EXPERIMENT 7:

	n	c (particles, $\times 10^6$/ml/min)	r	p
control, Hanks'	10	-0.00013	-0.706	2.5%>p>1.0%
5mM L-glutamine	4	-0.00151	-0.999	p = 0.05%
Eagles 50%, Hanks 50%	4	-0.00234	-0.993	0.25%>p>0.05%

EXPERIMENT 8:

control, Hanks'	7	-0.00013	-0.588	p > 5.0%
1mM L-glutamine	5	-0.00083	-0.983	0.25%>p>0.05%
5mM L-glutamine	5	-0.00068	-0.990	" p "
Eagles 50%, Hanks 50%, with 1mM L-glutamine	5	-0.00141	-0.982	" p "

D. Aggregation of L-glutamine treated cells

The rapidity of response of cells to L-glutamine was examined. This could not be done from experiments where an initial aggregation lag existed.

1) Methods

a) Experiment 9

In one experiment, normally harvested cells were resuspended in either Hanks or 4.63 mM L-glutamine in Hanks. The suspensions were aggregated in duplicate at 37°C.

b) Experiment 10

In one experiment normally harvested cells were resuspended as above in either Hanks or 5 mM L-glutamine. Aggregation was at 37°C. In another flask, cells were aggregated in 4 mls. of Hanks for 30 minutes. At that time, 0.1 ml. of Hanks was added containing L-glutamine to give a final concentration of 5 mM.

2) Results

In these experiments the initial lag in response to L-glutamine was eliminated and, at least as can be resolved here, the effect of the amino acid is practically instantaneous. It is interesting that, since L-glutamine promoted aggregation is a complicated metabolic phenomenon, the cellular response is as rapid as it appears.

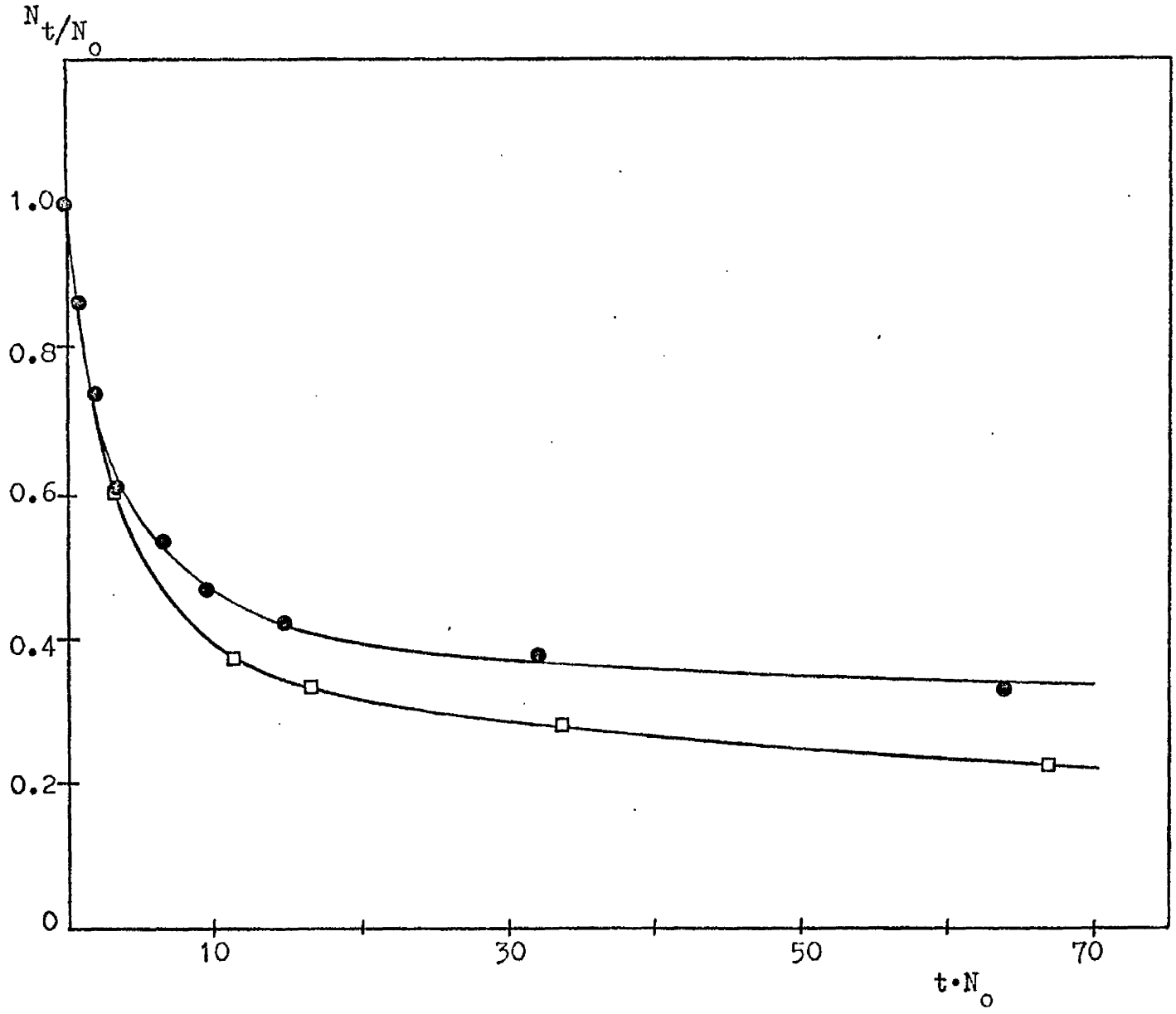


Figure 40. RAPID RESPONSE OF C-13 CELL AGGREGATION TO L-GLUTAMINE, exp. 9:

● Hanks control (average of duplicate flasks),
□ glutamine " added at t = 0, final concentration: 4.63mM.

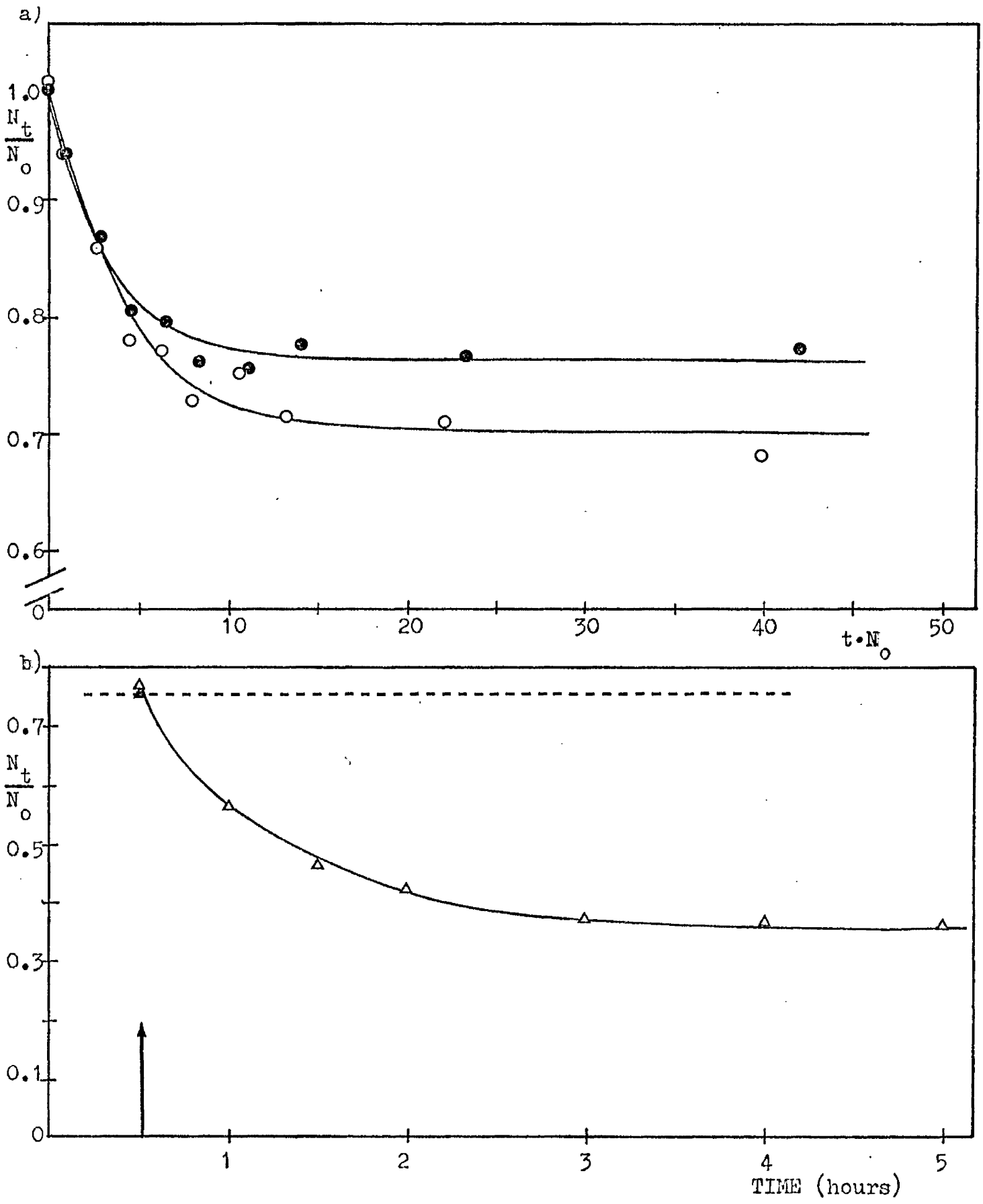


Figure 41.a)RAPID RESPONSE OF C-13 CELL AGGREGATION TO L-GLUTAMINE, exp. 10:
● Hanks control; ○ glutamine added at t = 0; final concentration: 5mM.
b)ADDITION OF L-GLUTAMINE TO PRE-FORMED C-13 CELL AGGREGATES, exp.10:
▲ Hanks control; △ glutamine added at t = 30; final concentration: 5mM.

TABLE 14

EFFECT OF L-GLUTAMINE ON C-13 CELL AGGREGATION

The concentration and time of addition of glutamine to the cell suspension is given. See text for details.

EXPERIMENT 10:

control, Hanks;

n	No	A	α	ϕ	Residual Variance	SEp
10	0.9326 ±0.0122	0.3305 ±0.0055	0.3082 ±0.0032	0.2359 ±0.0128	0.0001161	0.0108

5mM glutamine/ml. at t = 0';

10	0.8893 ±0.0178	0.2505 ±0.0099	0.2228 ±0.0076	0.2977 ±0.0173	0.0002231	0.0149

5mM glutamine/ml. at t = 30';

*10 0.875 - after 30': $c = -0.00121 \times 10^6$ particles/min., $r = -0.965$ (0.05% > p)

* Linear regression, see p 110

V. CONCLUSIONS

A. Interpretation of the Aggregation Parameters

The aggregation of C-13 cells in an agitated suspension, measured in terms of the total particle concentration, can be conveniently fitted to an exponential decay curve specified by the parameters A and ϕ. This simple treatment can be used when the aggregation medium is composed of glucose and salts; but when complex media are used aggregation is complicated by an additional process, probably metabolic, and a different treatment must be applied. Two questions require discussion: how do the parameters relate to the criteria of adhesiveness used by other workers, and what are the implications of the limited aggregation and the changes in the parameters caused by treatments with NANase, trypsin and L-glutamine?

A precise interpretation of ϕ depends on an understanding of why net aggregation stops. The limited aggregation in C-13 suspensions may be due to one of two phenomena. 1) Adhesiveness of the cell surface is uniform over each cell in the population and ϕ represents a situation where there is an equilibrium between the break-up of aggregates by shear and their reformation by aggregation. 2) There is an uneven distribution of adhesiveness in the population and, perhaps geometrically, on the surface of adhesive cells.

If/

If 1) is the case, the value of ϕ will be a function of the ratio of the rates of aggregate formation and break-up. An increase in ϕ may mean a uniform increase in adhesiveness caused either by an increase in the collision efficiency of formation of adhesions or by a decrease in the probability of break-up of cell clusters. However, this case appears unlikely with C-13 cells since no cluster break-up occurs when an aggregated suspension is cooled to $0^{o}c$ (Edwards and Campbell 1971a). On the other hand, in the case of 2), an increase in ϕ can indicate an increase in the fraction of adhesive cells or cell surface in the population. The concept of adhesive heterogeneity on the cell surface is reasonable on three accounts. First, all the identified cell junctions, with the exception of the intermediate junction, occur over limited areas of the cell surface. Secondly, Edwards and Campbell (1971a) have mentioned the possibility that specially adhesive "patches" could originate from the sites of pre-existing intercellular contacts formed in culture. Thirdly, Gerisch (1968) suggested that the sites of adhesive surface in slug-forming slime mold cells were unevenly distributed spatially on the cell surface.

An increase in ϕ indicates that more adhesions are being formed and, in practice, in the C-13 system, is accompanied by the appearance of larger aggregates. As discussed previously, several /

several workers have used the maximum size of aggregates as a measure of adhesiveness which could relate to $\emptyset$ but need not necessarily do so. This depends on the shape of the particle size distribution. For example, if every cell in a suspension rapidly formed but one adhesion, then each aggregate would be no larger than two cells at maximum. The cells would be considered to be adhesive by the criteria of $\emptyset$ and of the rate constant A as well; but since no large aggregates are found the cells might be considered, incorrectly, to be non-adhesive by the size criterion. The same argument applies to actual systems which have been studied. The final number of cells in the larger aggregates, measured after long times in shakers, may have little relationship with the probability of adhesion or the proportion of adhesive cells or cell surface in the population. The rate constant A is used to compare the values between suspensions of slightly differing N_o since, when a given suspension is aggregated from different initial particle concentrations, the values of α are (at least approximately) proportional to N_o. This is a consequence of the collision nature of the process. It is not necessarily obeyed to sufficient accuracy to be used to compare suspensions with largely differing values of N_o (Edwards and Campbell 1971a). In virtually every experiment where aggregation, as measured by $\emptyset$, had been either enhanced (by NANase or metabolic generation) or decreased (by trypsin) the value of A remained either unchanged or only slightly, but inconsistently, altered.

It should be emphasized that where $\emptyset$ increased or decreased and A remained constant, then the rate of aggregation, dN_t/dt, was also either increased or decreased respectively. This observation leaves open the possibility that the collision efficiency of the adhesive surface in the population remains unchanged from the control when $\emptyset$ is altered. If this is so, then the aggregation rate changes because the relative or absolute concentration of such adhesive surface in the population either increases or decreases as a result of the treatment.

Curtis and Greaves (1965) and Curtis (1969) have suggested that the rate of aggregation, or more correctly, the collision efficiency, is a function of the strength of intercellular adhesion. On this basis, the observations that A is only marginally affected, if at all, by experimental treatments which cause large changes in $\emptyset$ could be held to indicate that the strength of intercellular adhesion (in this system) is not affected by the treatments. If uneven geometry of adhesive surface is a feature of C-13 cells then the rate constant A must be a complex function of both the "patch" collision rate (which depends on patch size and concentration) and on the strength of patch adhesion, i.e. collision efficiency of patches. Collisions involving only non-adhesive surface on each cell will not result in adhesion. Therefore, the

larger the fraction of non-adhesive surface per adhesive cell, the lower will be the values of the average collision efficiency and of A although the collision efficiency of collisions involving patches will remain constant.

If A is a direct function of the proportion of adhesive surface per adhesive cell, then the apparent collision efficiency will be underestimated if this proportion is low. The kinetic interpretation is made more difficult if adhesive "patches" adhere not only to themselves but to otherwise non-adhesive cell surface as well. Thus the use of the value of A to determine the relative adhesiveness of such cell suspensions may be of limited usefulness.

B. General Discussion

It has been found that trypsin and pronase are active in dispersing cells in culture and in aggregates and that trypsin decreases the adhesiveness of the cells (as measured by $\emptyset$ and A). Neuraminidase, on the other hand, increases aggregation by as much as 50% (although the magnitude of this figure may be limited by the hydrodynamic conditions in the system). L-glutamine was found to be the only component of complex /

complex media active in increasing intercellular adhesiveness This probably reflects a glutamine requirement for the synthesis and incorporation of new plasma membrane into the cell surface.

In addition, it was found that neither NANase treated nor control cells aggregated at 0°C and that adhesiveness promoted by NANase (at 37°C) was sensitive to trypsin. The effect of NANase treatment on aggregation was proportionately greater if lower growth density cells were used. EDTA, collagenase and phospholipase C had no significant effect on C-13 aggregation as tested here.

The observations of the effect of trypsin on cell aggregation indicate that the limited extent of aggregation does not depend on patches of the cell surface which are protected from trypsin merely by intercellular contact. It is concluded that the trypsin sensitive adhesive surface also exists in the culture before the cells are harvested or treated with the enzyme. Trypsin may function in decreasing adhesiveness by removing proteins or glycoproteins from the cell surface and thus either: a) removing the cell surface adhesive components, or b) exposing charged groups beneath the "protein layer". If the ζ potential of the cells is increased sufficiently, cell contact may be inhibited by the greater electrostatic repulsive force. As an example of b), the

treatment /

treatment of cat erythrocytes with proteases results in an increase in their electrophoretic mobility (Uhlenbruck et al 1968b).

NANase, by removing all or most of the cell surface NANA residues, may increase cellular adhesion in one of two ways: 1) by exposing new terminal groups on the sugar chains of glycolipids and/or glycoproteins (possibly β-galactosyl and/or N-acetyl-β-D-galactosamine) to which NANA forms an α-glycosidic bond (Pardoe et al, 1970). The new terminal residues could be the determinants of cell surface adhesive components; 2) by removing NANA and thus substantially decreasing the cell surface ζ potential allowing closer cellular approach and greater adhesive contact consistent with the lyophobic colloid theory of cell adhesion (Curtis 1960).

The mechanism of adhesion of NANase treated C-13 cells cannot be differentiated from that causing the adhesion of untreated cells on the basis of the temperature and trypsin sensitivity of the aggregation.

The generation of adhesive cell surface by complex media probably reflects a requirement for protein and amino-sugars in cell surface molecules important in adhesion. The dependence does not mean that a particular amino-sugar is an adhesive determinant on C-13 cells. The synthesis of the sugar /

sugar portions of cell surface glycolipids and glycoproteins depends on specific glycosyl transferases linking nucleotide sugars to specific acceptors, i.e. the correct, incomplete, sugar chain (Roseman 1970, but see Meezan et al 1969).

If an early residue in the chain is not available, or if the transferase is lacking or defective, either the chain will remain incomplete at that point when incorporated into the cell surface or, possibly, cell surface synthesis and incorporation may be inhibited. The generation of adhesive surface by L-glutamine treated cells or by heavily trypsinized cells incubated in complex media probably reflects the process of plasma membrane turnover and not that of piecemeal repair of the adhesive and trypsin sensitive components alone (Kraemer 1967a, Warren and Glick 1968, see Pasternak and Bergeron 1970).

Roseman (1970) has described the function of cell surface galactosyl acceptors in the adhesion of chick embryonic neural retinal cells. In this system, the galactosyl acceptors may be functionally analogous to the neuraminyl acceptors on the C-13 cell surface. The neural retinal cell adhesion is believed to be through a complementary mechanism, as discussed previously, which requires two different components on the cell surface. In the neural retinal system, the second component is believed to be the cell surface galactosyl transferase enzyme /

enzyme binding to the incomplete galactosyl acceptor (Roseman 1970). Two components, one sugar and the other protein, are also suggested to be required for bacteria-phage adhesion. Robbins and Uchida (1962) have found that a particular Salmonella phage binds to the bacteria by the specific interaction of viral protein tail fibers and a certain cell surface sugar chain. The mechanism of binding is unknown.

Of particular interest are the observations of the effect of NANase on cell-antibody and -lectin interactions and macrophage-bacteria binding.

Uhlenbruck and Prokop (1967) found that the treatment of cells with NANase allowed normally present serum antibodies to bind to the cells. There was no detectable binding of antibody by the cells before the enzyme treatment. The authors proposed that the antibodies became effective because NANase exposed new antigens, known as Freidenriech antigens, on the cell surface and not because of a defect in the antibody molecules.

One of these new determinants was identified as a β-galactosyl residue and known as the T antigen. It was the site of binding of NANA to the sugar chain on the cell surface. The specific lectin of Ricinus communis was also bound by cell surface β-galactosyl groups after NANase treatment (Pardoe et /

et al 1970). Uhlenbruck and Prokop (1967) suggested that the binding of the antibodies to the NANase treated cells was due to both; a) the effect of the decrease in the cell surface net negative charge which might allow closer approach of antibodies to cell surface glycoprotein and glycolipid determinants, and b) the exposure of a new terminal group(s) once NANA is removed.

In regard to the functions of cell surface charge in cellular interactions, Currie and Bagshawe (1967, 1968) have discussed the contribution of NANA to the cell surface charge of the trophoblast and various types of malignant cells. The authors suggested that the magnitude of this charge controls the interaction of these cells with antibodies and with other cells.

Thus these phenomena have some analogy with the effect of NANase on C-13 aggregation. A closer analogy has been observed with the effect of NANase and trypsin on the attachment phase of phagocytosis of bacteria by macrophages.

Allen and Cook (1970) have found that Bacillus sps. opsonized with a component of calf serum (probably a 19S macroglobulin) are able to adhere to the macrophage cell surface. The adhesion is greatly increased (by about 50%) if the cells are pre-treated with NANase. Trypsin and pronase can abolish /

abolish the adhesion but the phospholipases A and C have no effect.

The authors observed that after trypsin treatment, the adhesive capacity of the cultured macrophages regenerated; and that the regeneration was inhibited by an analogue of L-glutamine, azaserine.

These five features of the phenomenon are strikingly parallel with those observed for C-13 aggregation. Allen and Cook (1970) suggested that the removal of cell surface NANA residues enhanced the adhesion by causing a change in the configuration of the plasma membrane which re-arranged the cell surface adhesive components into more favourable positions. Trypsin and pronase were suggested to function by decreasing the number of cell surface bacterial-opsonin receptor groups.

A simpler explanation for the effect of NANase of C-13 aggregation, also applicable to the macrophage-bacteria phenomenon, could be that the treatment exposes adhesive molecules which were formerly blocked by NANA residues (as with the T antigen). Alternatively, C-13 adhesion may be increased by the reduction of repulsive electrostatic force between cells once NANA residues are removed from their surface. Both these explanations fit my own observations, but the lack of effect of EDTA on C-13 aggregation fails to support the latter conclusion.

The/

The conclusions by Allen and Cook (1970) concerning the effect of trypsin and pronase on the macrophage-bacteria binding could also hold for the effect of these enzymes on C-13 adhesion.

C. Conclusions

Although it is possible that the effects of trypsin, pronase and NANase on C-13 aggregation may be interpreted in purely electrostatic terms, the studies in which adhesiveness is regenerated must focus attention on the possibility that the adhesion of the dispersed cells depends on specific cell surface molecules. In particular, it appears that sugar molecules (and probably their polysaccharide structure) of glycoproteins, and perhaps also glycolipids, are important in this regard. In the case of NANase, it should be possible to distinguish the electrostatic and specific molecule explanations by further exposing the NANase treated cells to enzymes selected to attack the new terminal sugar residues of the NANA acceptors.

If there is specific recognition of these sugars, there must be a second class of adhesive molecule on the cell surface which is complementary to the sugar adhesive determinants.

The /

The galactosyl transferase and the viral tail fibers have been suggested to be complementary components with binding functions (see above). However, a complementary protein component important in adhesion need not be an enzyme.

The postulated cell surface sugar requirement of C-13 aggregation is a feature which is reminiscent of the predominant role played by cell surface sugar residues in immune interactions, and the interactions of cells with virus and with bacteria. They may also have a vital role in immunological recognition (Burnet 1961, Shen and Ginsberg 1968) and morphogenetic differences (see Hakamori et al 1967).

In the future it will be important to attempt to relate the features of aggregation of dispersed cells in suspension (clearly a very artificial situation) to intercellular adhesion and the control of cell behaviour in the animal. Of special interest in this context are the observations on the relation of adhesiveness and growth density (Edwards and Campbell 1971a), and the abolition of aggregation caused by transformation of C-13 cells with polyoma virus (Edwards and Campbell 1971b).

The type of systematic investigation of adhesion described for C-13 cells by these authors and in this work should be readily applicable to other cell types and cell lines./

lines. The numerous biochemical, cytogenetic, behavioural and immunochemical investigations being carried out on cell lines, such as C-13, make them ideal for study since it may be possible to relate cell adhesion and cell behaviour and to describe both in more chemical terms.

APPENDIX I : MATERIALS

1. 120 cm^2 GLASS BOTTLES :	Flow Laboratories Ltd. washed with detergent and soaked overnight in concentrated sodium hypochlorite solution (British Drug Houses Ltd.) after use. Sterilized by heating at 160°C/2 hours in an oven.
2. 75cm^2 PLASTIC BOTTLES :	Falcon Plastics Inc.,U.S.A.
3. MODIFIED EAGLES MEDIUM :	(Stoker and Macpherson 1964) obtained from The Institute of Virology, Glasgow.
4. CALF SERUM :	Flow Laboratories Ltd.
5. TRYPTOSE PHOSPHATE BROTH (TPB) :	Difco Ltd.
6. 5% CO_2 :	5% CO_2, 95% air "Medical mixture", sterile. British Oxygen Corporation Ltd.
7. "TRIS" SOLUTION pH 7.4 :	Trizma base, (tris hydroxymethyl aminomethane) Sigma Ltd., 25mM NaCl, British Drug Houses, 140mM KCl, British Drug Houses, 5mM Na_2PO_4, British Drug Houses, 0.7mM. (in glass distilled H_2O micropore sterilized, all components Analar grade or equivalent).

8. /

8. 0.05% TRYPSIN-EDTA pH 7.4 :	3 volumes of trypsin in tris added to 2 volumes of EDTA in tris. Trypsin (Difco 1:250) 6.33 mg/ml. prepared by dissolving the powder in ice cold tris and then centrifugation at 600g for 1 hour to remove undissolved particles. 0.55mM EDTA (ethylenediamine tetra-acetate, disodium salt) British Drug Houses Ltd. AnalaR. Dissolved in tris. Solution sterilized by micropore filtration at 0°C. Stored at -20°C.
9. HANKS SOLUTION : TITRATION TO pH 7.2 WITH 6N HCl, AnalaR.	NaCl, 8g. ; KCl, 0.4 g $Na(CO_3)_2$, 0.35 g; KH_2PO_4, 0.5g $CaCl_2.2H_2O$, 0.185g; $MgSO_4.7H_2O$, 0.1 g . $MgCl_2.6H_2O$, 0.1g glucose, 5g ; tris, 3g phenol red 0.5%, 1ml, Flow Ltd. (per liter glass distilled H_2O, micropore sterilized, all components AnalaR or equivalent).
10. SILICON FLUID :	Hopkins and Williams Ltd. M51107, 0.1% in ethyl acetate AnalaR.
11. COULTER COUNTER, MODEL A :	Coulter Electronics Ltd.
12. MICROPIPET :	Eppendorf, Germany.
13. PURIFIED TRYPSIN :	Sigma Chemical Co.Ltd., type III. from bovine pancreas, 2 x cryst.,dialyzed 10,000 BAEE units/mg.
14. DNAase :	Sigma Chemical Co.Ltd., DN-C type I from bovine pancreas, 1x cryst. 2030 Kunitz units/mg.
15. TRYPSIN INHIBITOR (ti) :	Sigma Chemical Co.Ltd., type 1 - S from soya bean, one mg. inhibits 1.6 mg. trypsin.

16. /

16. PURIFIED COLLAGENASE : Sigma Ltd., type III fraction A activity 1 mg. releases the equivalent of 225 μM Leucine in 18 hours pH 7.4, 37°C.

17. PURIFIED TRYPSIN : Worthington Biochemical Corporation, U.S.A. 2x cryst. approximately 10,000 BAEE units/mg.

18. PRONASE : Sigma Chemical Co.Ltd. type IV. Streptomyces griseus protease activity, 1 mg. will liberate approximately 4.0 μM tyrosine/min from casein at pH 7.5, 37°C.

19. AUTOMATIC TITRATOR : Radiometer, Denmark titrator TTT1C, titrigraph SBR2C

20. L-α-LECITHIN : Sigma Chemical Co.Ltd., type III-E egg yolk, chromatographically prepared, hexane solution.

21. TETRAHYDROFURAN (THF) : British Drug Houses Ltd.

22. $CaCl_2$-$2H_2O$: British Drug Houses Ltd., AnalaR.

23. NITROGEN : British Oxygen Corporation Ltd., white spot grade.

24. PHOSPHOLIPASE C : Sigma Chemical Co.Ltd., type I Clostridium welchii activity; 1 mg. liberates 3.7 μM water soluble organic phosphorus at pH 7.3, 37°C from egg yolk lecithin.

25. NaOH : British Drug Houses Ltd., AnalaR.

26. HCl : British Drug Houses Ltd., AnalaR.

27. /

27.	NEURAMINIDASE (NANase) :	Sigma Ltd: type VI chromatographically purified from Clostridium perfringens activity; 1.1 units/mg. using neuramin-lactose, 0.75 units/mg. using Bovine Submexillary Mucin.
28.	PLASTIC PETRI DISHES :	Falcon Plastics Inc., U.S.A.
29.	GLUCOSE FREE HANKS (GFH) :	As in 9 but without glucose.
30.	PHOSPHATE BUFFER : pH 7.24	0.02 M . 0.2 M KH_2PO_4 37.5 vols, 0.2 M K_2HPO_4 85 vols, 10 mls. 90 mls. distilled H_2O all components AnalaR.
31.	NEURAMINIC ACID ALDOLASE :	Sigma Chemical Co.Ltd., grade III from Clostridium perfringens prepared in 0.02 M phosphate buffer, pH 7.2, activity; 0.75 units/mg. protein (80% protein)
32.	LACTATE DEHYDROGENASE (LDH) :	Sigma Chemical Co.Ltd., type II from rabbit muscle cryst. in saturated ammonium sulfate activity; 1 mg. will convert 500 μM pyruvate to lactate/min. pH 7.5, 37°C.
33.	NADH :	Sigma Chemical Co.Ltd., grade III disodium salt.
34.	MICROPORE FILTERS:	Millipore Ltd.
35.	FLUORIMETER :	Locarte Ltd., model MK-5.
36.	PYRUVIC ACID :	Sigma Chemical Co.Ltd., type II. sodium salt.
37.	N-ACETYL-NEURAMINIC ACID :	Sigma Chemical Co.Ltd., type IV cryst. synthetic.
38.	MEDIUM 199 :	Flow Laboratories Ltd.

39.	HEPES :	Sigma Chemical Co.Ltd.
40.	EAGLES MEDIUM VITAMINS:	from Institute of Virology, Glasgow. 250x concentrate stored frozen.
41.	EAGLES MEDIUM AMINO ACIDS: (LACKING L-GLUTAMINE)	from the Institute of Virology, Glasgow. 20x concentrate stored frozen.
42.	N-ACETYL-D-GLUCOSAMINE :	Sigma Chemical Co.Ltd.
43.	N-ACETYL-D-MANNOSAMINE - H_2O :	Sigma Chemical Co.Ltd.
44.	D (+) GALACTOSAMINE HCl :	Sigma Chemical Co.Ltd.
45.	N-ACETYL-D-GALACTOSAMINE:	Sigma Chemical Co.Ltd., cryst.
46.	L-GLUTAMINE :	Sigma Chemical Co. Ltd., grade III cryst.
47.	AZASERINE :	(O-diazoacetyl-L-serine) Calbiochem Inc. U.S.A., grade A.
48.	L-GLUTAMIC ACID :	Sigma Chemical Co.Ltd.,free acid cryst.
49.	D (+) GALACTOSE :	Sigma Chemical Co.Ltd., Sigma grade cryst.
50.	α LACTOSE :	Sigma Chemical Co. Ltd., monohydrate cryst.
51.	D (+) MANNOSE :	Sigma Chemical Co.Ltd., cryst.
52.	α -L-and α -D-FUCOSE :	Sigma Chemical Co.Ltd.
53.	$(NH_4)_2 SO_4$:	British Drug Houses Ltd., AnalaR.
54.	(meso) INOSITOL :	British Drug Houses Ltd., AnalaR.

55. D (+) GLUCOSAMINE HCl :	Sigma Chemical Co.Ltd.
56. D-MANNOSAMINE HCl :	Sigma Chemical Co.Ltd.
57. N-ACETYL-NEURAMIN-LACTOSE :	Sigma Chemical Co.Ltd., type I free acid, from bovine colostrum.
58. NaF :	British Drug Houses Ltd. AnalaR.
59. TRIS BUFFER :	Tris 36.3g, Sigma Chemical Co.Ltd. IN HCl 48.0 ml, British Drug Houses Ltd.,AnalaR. TEMED 0.46 ml, (N,N,N,N - tetra methyl ethylene diamine) Eastman Kodak Ltd.; distilled H_2O, to 100 mls.
60. 7.5% ACRYLAMIDE :	Acrylamide, 30.0g, Eastman Kodak Ltd. Bis 0.8g, (N,N Methylene bis acrylamide) Eastman Kodak Ltd. $K_3Fe(CN)_6$ 0.015g, British Drug Houses Ltd., AnalaR, distilled H_2O, to 100 mls.
61. INITIATOR :	Ammonium persulfate 0.14g, British Drug Houses Ltd., AnalaR., distilled H_2O, to 100 mls.
62. 15% ACRYLAMIDE :	as in 60 but 60.0g acrylamide.
63. ELECTROPHORESIS APPARATUS :	Shandon Scientific Co.Ltd.
64. RESERVOIR BUFFER :	Glycine 28.8g, Sigma Ltd. tris 6.0g, Sigma Ltd. distilled H_2O to 1 liter.
65. SUCROSE :	Sigma Ltd. cryst. in tris buffer pH 9.5.

66. /

66. BROMOPHENOL BLUE : British Drug Houses Ltd.

67. ACETIC ACID : British Drug Houses Ltd. glacial, Analar.

68. AMIDO BIACK : (naphthalene black) British Drug Houses Ltd.

APPENDIX II

Gel Electrophoresis of Neuraminidase and Phospholipase C.

A. Preparation of acrylamide gels

Gels of 7.5% and 15% acrylamide were prepared at pH 9.5.

1) The 7.5% gel consisted of a tris buffer[59], one vol.; acrylamide[60], 2 vols.; ammonium persulfate initiator[61], 4 vols.; and one volume of de-aerated, distilled H_2O.

2) The 15% gel contained 2 volumes of acrylamide[62]. The buffer, initiator and H_2O was as before.

Before polymerization in glass tubes each mixture was degassed and covered with H_2O. Once polymerized the water layer was removed and the gels were placed in a Shannon disc electrophoresis apparatus[63] and layered with the reservoir glycine-tris buffer solution[64]. 50μl of a 20% sucrose solution, pH 9.5[65], containing the sample was carefully layered under this buffer. The concentrations of the added proteins were: phl C, 8.7 mg/ml.; NANase, 3.2 mg/ml.

B. Electrophoresis

The upper and lower reservoirs were then filled with the reservoir buffer and 1 ml. of 0.001% bromophenol blue[66] was added to the upper solution. The electrophoresis was carried out at 200 volts, and 5mA/tube, for about one hour.

The/

The gels were removed in 7% acetic acid[67] and stained in 0.3% amido black[68] for one hour. De-staining was by immersion in several changes of 7% acetic acid.

C. Results

Photographs of the gels are shown in fig. 42 and 43 . Each enzyme preparation contains a number of proteins which can be detected by this method.

bands:

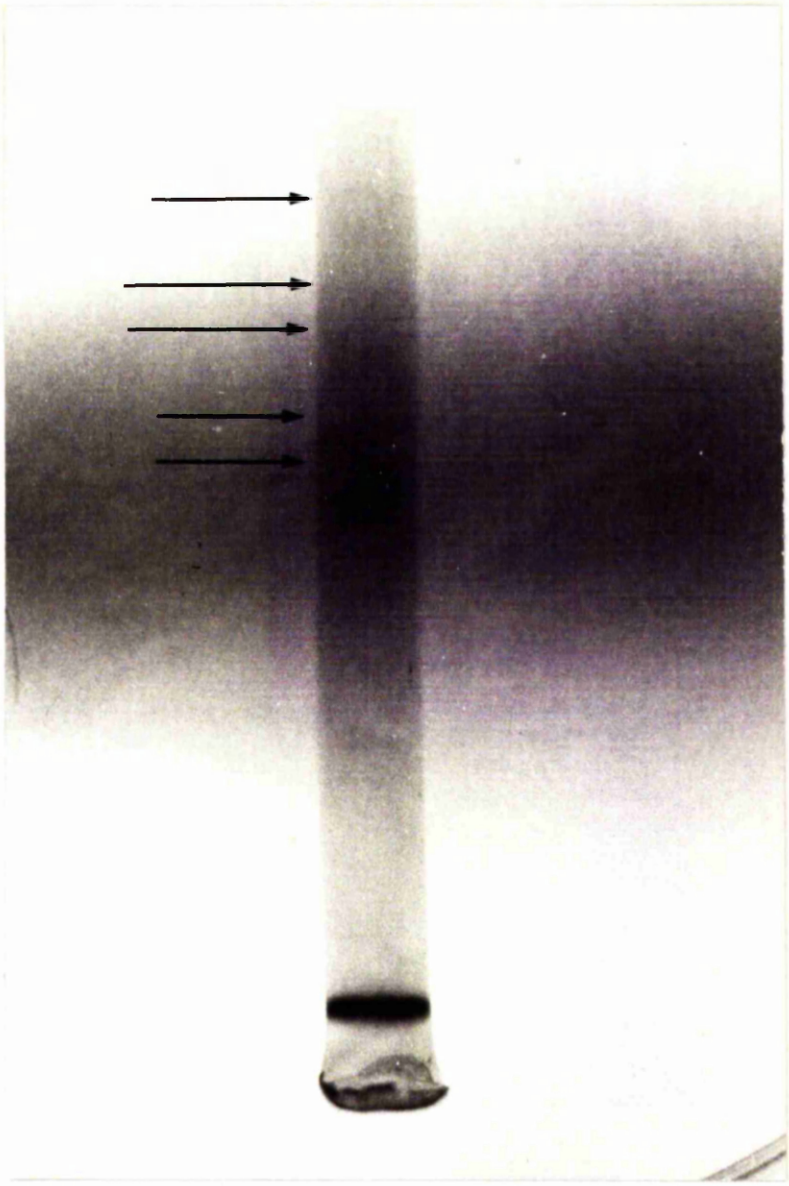
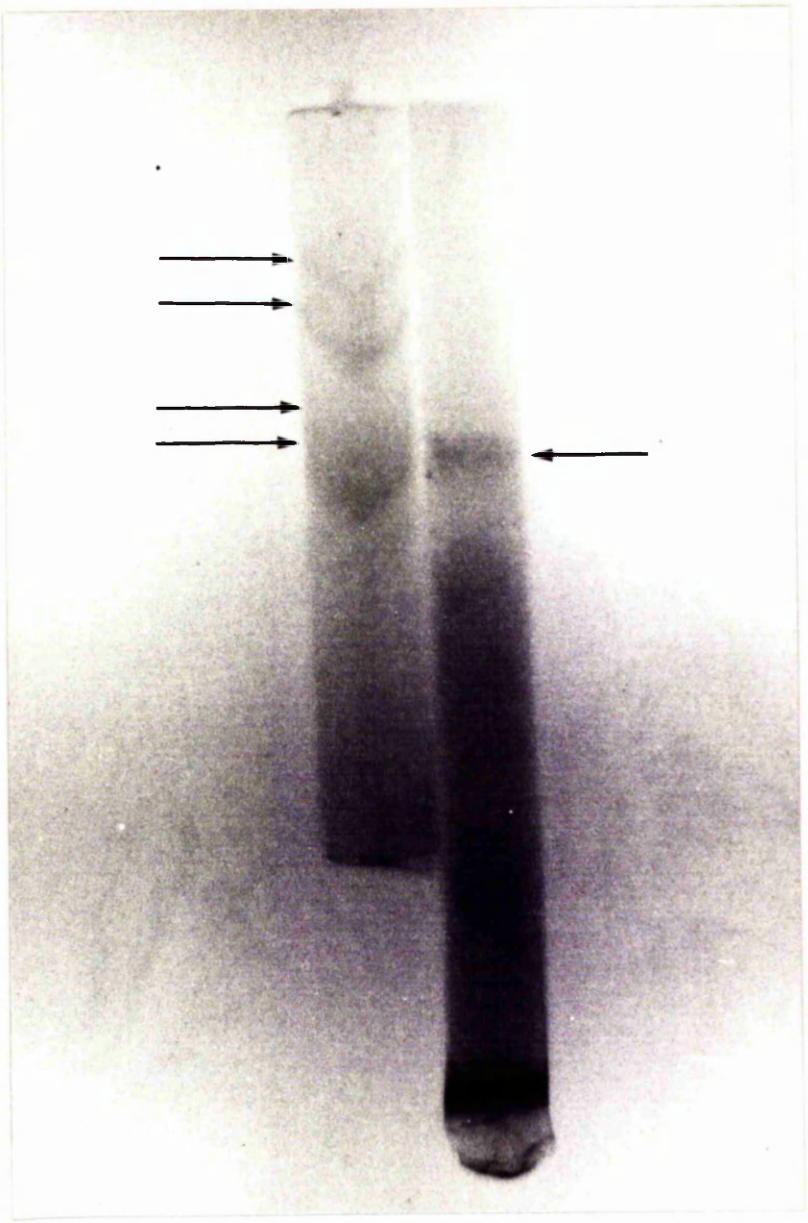


Figure 42.

Gel Electrophoresis of Phospholipase C, 7.5% acrylamide. Five protein bands can be seen in the gel and these are indicated by arrows.

Magnification: x2

bands:



bands:

% acrylamide: 15% 7.5%

Figure 43.
Gel Electrophoresis of Neuraminidase as indicated. Five protein bands can be distinguished (arrows).

Magnification: x2

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